(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 March 2003 (20.03.2003)

PCT

(10) International Publication Number
WO 03/023048 A2

(51) International Patent Classification[7]: C12Q

(21) International Application Number: PCT/US02/28410

(22) International Filing Date: 6 September 2002 (06.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/317,653 6 September 2001 (06.09.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/317,653 (CIP)
Filed on 6 September 2001 (06.09.2001)

(71) Applicant *(for all designated States except US)*: **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PETERSON, Randall** [US/US]; 42 Perkins Street, Stoneham, MA 02180 (US). **FISHMAN, Mark, C.** [US/US]; 43 Kenwood Avenue, Newton Center, MA 02459 (US).

(74) Agent: **MICHAUD, Susan, M.**; Clark & Elbing LLP, 101 Federal Street, Boston, MA 02110 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/023048 A2

(54) **Title:** METHODS FOR DIAGNOSING AND TREATING DISEASES AND CONDITIONS ASSOCIATED WITH PROTEIN KINASE Cλ

(57) **Abstract:** The invention provides methods of diagnosing diseases and conditions associated with PKCλ, methods for identifying compounds that can be used to treat or to prevent such diseases and conditions, and methods of using these compounds to treat or to prevent such diseases and conditions. Also provided in the invention are animal model systems that can be used in screening methods.

# METHODS FOR DIAGNOSING AND TREATING DISEASES AND CONDITIONS ASSOCIATED WITH PROTEIN KINASE C λ

## Field of the Invention

This invention relates to methods for diagnosing and treating diseases and conditions associated with Protein Kinase C λ.

## Background of the Invention

The processes by which organs acquire global structures and patterns during development are highly complex, and likely involve multiple, overlapping biochemical pathways. In the vertebrate heart, for example, the first key visible step in this process is chamber morphogenesis, involving the fashioning of the atrium and the ventricle. Proper orientation of these two functionally distinct contractile units is required for unidirectional blood flow, which begins with the first heartbeat of an organism. Properly formed chambers thereafter are the substrates upon which further heart development is superimposed.

Over recent years, much has been learned about the molecular mechanisms that are responsible for the acquisition of characteristic atrial and ventricular cell fates (Fishman et al., Development 124:2099-2117, 1997; Srivastava et al., Nature 407:221-226, 2000). However, both embryological and molecular steps that fashion the higher order structures of these chambers have proven to be more elusive because, in part, unlike cell fate decisions, these steps can be studied meaningfully only in living organisms. The zebrafish, *Danio rerio*, is a convenient organism to use in genetic and biochemical analyses of development. It has an accessible and transparent embryo, allowing direct observation of organ function from the earliest stages of development, has a short generation time, and is fecund.

## Summary of the Invention

The invention provides diagnostic, drug screening, and therapeutic methods that are based on the observation that a mutation, designated the "*heart and soul* (*has*)"

mutation, in the zebrafish Protein Kinase C λ (PKCλ) gene, as well as a small molecule identified in a chemical screen in zebrafish, concentramide, cause abnormal heart growth and development.

In a first aspect, the invention provides a method of determining whether a test subject (e.g., a mammal, such as a human) has or is at risk of developing a disease or condition related to PKCλ (e.g., a disease or condition of the heart; also see below). This method involves analyzing a nucleic acid molecule of a sample from the test subject to determine whether the test subject has a mutation (e.g., the *has* mutation; see below) in a gene encoding PKCλ. The presence of such a mutation indicates that the test subject has or is at risk of developing a disease related to PKCλ. This method can also involve the step of using nucleic acid molecule primers specific for a gene encoding PKCλ for nucleic acid molecule amplification of the gene by the polymerase chain reaction. It can further involve sequencing a nucleic acid molecule encoding PKCλ from a test subject.

In a second aspect, the invention provides a method for identifying compounds that can be used to treat or prevent a disease or condition associated with PKCλ, or in the preparation of a medicament for use in such methods. This method involves contacting an organism (e.g., a zebrafish) having a mutation in a PKCλ gene (e.g., the *heart and soul* mutation), and having a phenotype characteristic of such a disease or condition, with the compound, and determining the effect of the compound on the phenotype. Detection of an improvement in the phenotype indicates the identification of a compound that can be used to treat or prevent the disease or condition. In a variation of this method, the organism, with or without a mutation in the PKCλ gene (e.g., the *has* mutation), is contacted with a candidate compound in the presence of concentramide.

In a third aspect, the invention provides a method of treating or preventing a disease or condition related to PKCλ in a patient (e.g., a patient having a mutation (e.g., the *heart and soul* mutation) in a PKCλ gene), involving administering to the patient a compound identified using the method described above. Also included in the invention is the use of such compounds in the treatment or prevention of such diseases or conditions, as well as the use of these compounds in the preparation of medicaments for such treatment or prevention.

In a fourth aspect, the invention provides an additional method of treating or preventing a disease or condition related to PKCλ in a patient. This method involves administering to the patient a functional PKCλ protein or a nucleic acid molecule (in,

e.g., an expression vector) encoding the protein. Also included in the invention is the use of such proteins or nucleic acid molecules in the treatment or prevention of such diseases or conditions, as well as the use of these proteins or nucleic acid molecules in the preparation of medicaments for such treatment or prevention.

In a fifth aspect, the invention includes a substantially pure zebrafish PKCλ polypeptide. This polypeptide can include or consist essentially of, for example, an amino acid sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2. The invention also includes variants of these polypeptides that include sequences that are at least 75%, 85%, or 95% identical to the sequences of these polypeptides, and which have PKCλ activity or otherwise are characteristic of the diseases and conditions mentioned elsewhere herein. Fragments of these polypeptides are also included in the invention. For example, fragments that include any of the different domains of PKCλ, in varying combinations, are included.

In a sixth aspect, the invention provides an isolated nucleic acid molecule (e.g., a DNA molecule) including a sequence encoding a zebrafish PKCλ polypeptide. This nucleic acid molecule can encode a polypeptide including or consisting essentially of an amino sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2. The invention also includes nucleic acid molecules that hybridize to the complement of SEQ ID NO:1 under highly stringent conditions and encode polypeptides that have PKCλ activity or otherwise are characteristic of the diseases and conditions mentioned elsewhere herein.

In a seventh aspect, the invention provides a vector including the nucleic acid molecule described above.

In an eighth aspect, the invention includes a cell including the vector described above.

In a ninth aspect, the invention provides a non-human transgenic animal (e.g., a zebrafish or a mouse) including the nucleic acid molecule described above.

In a tenth aspect, the invention provides a non-human animal having a knockout mutation in one or both alleles encoding a PKCλ polypeptide.

In an eleventh aspect, the invention includes a cell from the non-human knockout animal described above.

In a twelfth aspect, the invention includes a non-human transgenic animal (e.g., a zebrafish) including a nucleic acid molecule encoding a mutant PKCλ polypeptide, e.g., a polypeptide having the *heart and soul* mutation.

In a thirteenth aspect, the invention provides an antibody that specifically binds to a PKCλ polypeptide.

By "polypeptide" or "polypeptide fragment" is meant a chain of two or more (e.g., 10, 15, 20, 30, 50, 100, or 200, or more) amino acids, regardless of any post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally or non-naturally occurring polypeptide. By "post-translational modification" is meant any change to a polypeptide or polypeptide fragment during or after synthesis. Post-translational modifications can be produced naturally (such as during synthesis within a cell) or generated artificially (such as by recombinant or chemical means). A "protein" can be made up of one or more polypeptides.

By "Protein Kinase C λ protein," "Protein Kinase C λ polypeptide," "PKCλ protein," or "PKCλ polypeptide" is meant a polypeptide that has at least 45%, preferably at least 60%, more preferably at least 75%, and most preferably at least 90% amino acid sequence identity to the sequence of a human (SEQ ID NO:5) or a zebrafish (SEQ ID NO:2) PKCλ polypeptide. Polypeptide products from splice variants of PKCλ gene sequences and PKCλ genes containing mutations are also included in this definition. A PKCλ polypeptide as defined herein plays a role in heart development, modeling, and function. It can be used as a marker of diseases and conditions associated with PKCλ, such as heart disease (also see below).

By a "Protein Kinase C λ nucleic acid molecule" or "PKCλ nucleic acid molecule" is meant a nucleic acid molecule, such as a genomic DNA, cDNA, or RNA (e.g., mRNA) molecule, that encodes a PKCλ protein (e.g., a human (encoded by SEQ ID NO:4) or a zebrafish (encoded by SEQ ID NOs:1 or 3) PKCλ protein), a PKCλ polypeptide, or a portion thereof, as defined above. A mutation in a PKCλ nucleic acid molecule can be characterized, for example, by the insertion of a premature stop codon anywhere in the PKCλ gene. For example, codon R515 can be changed to a stop codon (CGA to TGA), or codon W519 can be changed to a stop codon (TGG to TAG). In addition to this zebrafish Protein Kinase C λ mutation (hereinafter referred to as "the *heart and soul* mutation"), the invention includes any mutation that results in aberrant PKCλ protein production or function, including, only as examples, null mutations and

additional mutations causing truncations. The truncations can be carboxyl terminal truncations in which the carboxyl terminal half of the protein (or a portion thereof) is not produced. For example, at least 10, 25, 50, 70, 75, 100, 150, 200, or 250 amino acids of the carboxyl terminal half of the protein can be absent.

The term "identity" is used herein to describe the relationship of the sequence of a particular nucleic acid molecule or polypeptide to the sequence of a reference molecule of the same type. For example, if a polypeptide or a nucleic acid molecule has the same amino acid or nucleotide residue at a given position, compared to a reference molecule to which it is aligned, there is said to be "identity" at that position. The level of sequence identity of a nucleic acid molecule or a polypeptide to a reference molecule is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs). These software programs match identical or similar sequences by assigning degrees of identity to various substitutions, deletions, or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

A nucleic acid molecule or polypeptide is said to be "substantially identical" to a reference molecule if it exhibits, over its entire length, at least 51%, preferably at least 55%, 60%, or 65%, and most preferably 75%, 85%, 90%, or 95% identity to the sequence of the reference molecule. For polypeptides, the length of comparison sequences is at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids. For nucleic acid molecules, the length of comparison sequences is at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably at least 110 nucleotides. Of course, the length of comparison can be any length up to and including full length.

A PKCλ nucleic acid molecule or a PKCλ polypeptide is "analyzed" or subject to "analysis" if a test procedure is carried out on it that allows the determination of its biological activity or whether it is wild type or mutated. For example, one can analyze

the PKCλ genes of an animal (e.g., a human or a zebrafish) by amplifying genomic DNA of the animal using the polymerase chain reaction, and then determining whether the amplified DNA contains a mutation, for example, the *heart and soul* mutation, by, e.g., nucleotide sequence or restriction fragment analysis.

By "probe" or "primer" is meant a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence (a "target"). The stability of the resulting hybrid depends upon the extent of the base pairing that occurs. This stability is affected by parameters such as the degree of complementarity between the probe and target molecule, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as the temperature, salt concentration, and concentration of organic molecules, such as formamide, and is determined by methods that are well known to those skilled in the art. Probes or primers specific for PKCλ nucleic acid molecules, preferably, have greater than 45% sequence identity, more preferably at least 55-75% sequence identity, still more preferably at least 75-85% sequence identity, yet more preferably at least 85-99% sequence identity, and most preferably 100% sequence identity to the sequences of human (SEQ ID NO:4) or zebrafish (SEQ ID NOs:1 and 3) PKCλ genes.

Probes can be detectably labeled, either radioactively or non-radioactively, by methods that are well known to those skilled in the art. Probes can be used for methods involving nucleic acid hybridization, such as nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, northern hybridization, *in situ* hybridization, electrophoretic mobility shift assay (EMSA), and other methods that are well known to those skilled in the art.

A molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, a cDNA molecule, a polypeptide, or an antibody, can be said to be "detectably-labeled" if it is marked in such a way that its presence can be directly identified in a sample. Methods for detectably labeling molecules are well known in the art and include, without limitation, radioactive labeling (e.g., with an isotope, such as $^{32}P$ or $^{35}S$) and nonradioactive labeling (e.g., with a fluorescent label, such as fluorescein).

By a "substantially pure polypeptide" is meant a polypeptide (or a fragment thereof) that has been separated from proteins and organic molecules that naturally accompany it. Typically, a polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a PKCλ polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure PKCλ polypeptide can be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid molecule encoding a PKCλ polypeptide, or by chemical synthesis. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A polypeptide is substantially free of naturally associated components when it is separated from those proteins and organic molecules that accompany it in its natural state. Thus, a protein that is chemically synthesized or produced in a cellular system that is different from the cell in which it is naturally produced is substantially free from its naturally associated components. Accordingly, substantially pure polypeptides not only include those that are derived from eukaryotic organisms, but also those synthesized in *E. coli*, other prokaryotes, or in other such systems.

By "isolated nucleic acid molecule" is meant a nucleic acid molecule that is removed from the environment in which it naturally occurs. For example, a naturally-occurring nucleic acid molecule present in the genome of cell or as part of a gene bank is not isolated, but the same molecule, separated from the remaining part of the genome, as a result of, e.g., a cloning event (amplification), is "isolated." Typically, an isolated nucleic acid molecule is free from nucleic acid regions (e.g., coding regions) with which it is immediately contiguous, at the 5' or 3' ends, in the naturally occurring genome. Such isolated nucleic acid molecules can be part of a vector or a composition and still be isolated, as such a vector or composition is not part of its natural environment.

An antibody is said to "specifically bind" to a polypeptide if it recognizes and binds to the polypeptide (e.g., a PKCλ polypeptide), but does not substantially recognize and bind to other molecules (e.g., non-PKCλ-related polypeptides) in a sample, e.g., a biological sample, which naturally includes the polypeptide.

By "high stringency conditions" is meant conditions that allow hybridization comparable with the hybridization that occurs using a DNA probe of at least 100, e.g., 200, 350, or 500, nucleotides in length, in a buffer containing 0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA (fraction V), at a temperature of 65°C, or a buffer containing 48% formamide, 4.8 x SSC, 0.2 M Tris-Cl, pH 7.6, 1 x Denhardt's solution, 10% dextran sulfate, and 0.1% SDS, at a temperature of 42°C. (These are typical conditions for high stringency northern or Southern hybridizations.) High stringency hybridization is also relied upon for the success of numerous techniques routinely performed by molecular biologists, such as high stringency PCR, DNA sequencing, single strand conformational polymorphism analysis, and *in situ* hybridization. In contrast to northern and Southern hybridizations, these techniques are usually performed with relatively short probes (e.g., usually 16 nucleotides or longer for PCR or sequencing, and 40 nucleotides or longer for *in situ* hybridization). The high stringency conditions used in these techniques are well known to those skilled in the art of molecular biology, and examples of them can be found, for example, in Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1998, which is hereby incorporated by reference.

By "sample" is meant a tissue biopsy, amniotic fluid, cell, blood, serum, urine, stool, or other specimen obtained from a patient or a test subject. The sample can be analyzed to detect a mutation in a PKCλ gene, or expression levels of a PKCλ gene, by methods that are known in the art. For example, methods such as sequencing, single-strand conformational polymorphism (SSCP) analysis, or restriction fragment length polymorphism (RFLP) analysis of PCR products derived from a patient sample can be used to detect a mutation in a PKCλ gene; ELISA and other immunoassays can be used to measure levels of a PKCλ polypeptide; and PCR can be used to measure the level of a PKCλ nucleic acid molecule.

By "Protein Kinase C λ-related disease," "PKCλ-related disease," "Protein Kinase C λ-related condition," or "PKC λ-related condition" is meant a disease or condition that results from inappropriately high or low expression of a PKCλ gene, or a mutation in a PKCλ gene (including control sequences, such as promoters) that alters the biological activity of a PKCλ nucleic acid molecule or polypeptide. PKCλ-related diseases and conditions can arise in any tissue in which PKCλ is expressed during

prenatal or post-natal life. PKCλ-related diseases and conditions can include diseases or conditions of the heart or cancer (also see below).

The invention provides several advantages. For example, using the diagnostic methods of the invention it is possible to detect an increased likelihood of diseases or conditions associated with PKCλ, such as diseases of the heart or cancer, in a patient, so that appropriate intervention can be instituted before any symptoms occur. This may be useful, for example, with patients in high-risk groups for such diseases or conditions. Also, the diagnostic methods of the invention facilitate determination of the etiology of such an existing disease or condition in a patient, so that an appropriate approach to treatment can be selected. In addition, the screening methods of the invention can be used to identify compounds that can be used to treat or to prevent these diseases or conditions. The invention can also be used to treat diseases or conditions (e.g., organ failure, such as heart or kidney failure) for which, prior to the invention, the only treatment was organ transplantation, which is limited by the availability of donor organs and the possibility of organ rejection.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

## Brief Description of the Drawings

Fig. 1A is a schematic representation of the structure of a small molecule, concentramide, that alters heart patterning.

Fig. 1B is a lateral view of the mushroom-shaped heart of a live, concentramide-treated embryo 30 hpf. The atrium is indicated with **A**, and the ventricle with **V**.

Fig. 1C is a schematic representation of a timecourse of concentramide effectiveness. Black bars indicate the developmental time periods during which groups of embryos were immersed in water containing concentramide. An "x" indicates that treatment during the indicated time period alters the wild-type brain or heart phenotypes. An "o" indicates that the wild-type phenotype was observed. Blue and pink boxes mark the critical periods for development of the brain and heart phenotypes, respectively.

Fig. 2 shows that hearts from *has* mutant embryos phenocopy hearts from concentramide-treated embryos. *In situ* hybridization was performed with wild-type (Figs. 2A-2C), concentramide-treated (Figs. 2D-2F), and *has* (Figs. 2G-2I) embryos. The expression pattern of cardiac myosin light chain 2 (cmlc2) is shown for embryos 24

hpf (Figs. 2A, 2D, and 2G) and 30 hpf (Figs. 2B, 2E, and 2H). The relative locations of atrium (A) and ventricle (V) were confirmed by 7 μm sagital sections of embryos in which the ventricle was prestained blue by *in situ* hybridization to ventricle-specific myosin heavy chain (vmhc), followed by staining of the atrium brown with the atrium-specific antibody S46 (Figs. 2C, 2F, and 2I). The view is dorsal, anterior up in Figs. 2A, 2D, and 2G. The view is lateral, anterior to the left in all other frames.

Fig. 3A is a map of the *has* interval with genomic structure of the zebrafish PKCλ gene. YAC and BAC clones are indicated by addresses beginning with "y" and "b." The BAC clone 23c14 was sequenced to determine the entire genomic structure of the *has* gene. From the partial sequence of the BACs listed, a preliminary transcript map of the region was determined (see Table 1). The zebrafish PKCλ gene comprises 18 exons represented by vertical lines. The site of the mutations associated with the m129 and m567 alleles is indicated with an asterisk.

Fig. 3B is an anti-PKCλ western blot of protein extracts from wild-type embryos (WT), *has* mutant embryos (m567 -/-), and siblings of *has* mutant embryos (m567 +/+ and +/-).

Figs. 3C-3E show that antisense disruption of PKCλ expression phenocopies the *has* mutation. Wild-type embryos (3C), *has* embryos (3D), and wild-type embryos injected with a PKCλ antisense morpholino oligomer (3E) were photographed live 2 days postfertilization.

Fig. 4 shows that PKCλ is required for lamination, cell polarity, and epithelial cell-cell interaction in the retina. Transverse 5 μm sections of wild-type (Figs. 4A-4B), concentramide-treated (Figs. 4C-4D), and *has* (Figs. 4E-4F) embryos were stained with hematoxylin-eosin 5 days postfertilization (Figs. 4A, 4C, and 4E) or with dapi 30 hpf (Figs. 4B, 4D, and 4F). Arrowheads indicate mitotic nuclei. Zonula occludens-1 localization in the retina is shown by 5 μm transverse sections following staining of wild-type (Fig. 4G) or *has* (Fig. 4H) embryos with an anti-ZO-1 antibody.

Fig. 5 shows the effects of PKCλ inactivation and concentramide treatment on polarity of the zebrafish kidney and the *C. elegans* embryo. An apical kidney marker (3G8) was used to stain kidneys of wild-type (Fig. 5A), concentramide-treated (Fig. 5B), and *has* (Fig. 5C) embryos. Transverse 2 μm sections of the pronephric duct are shown. Figs. 5D and 5E, *C. elegans* strain KK871, a stable expresser of a par2:GFP fusion

protein, was treated with 34 μM concentramide and allowed to develop at room temperature. Nomarski (Fig. 5D) and fluorescence (Fig. 5E) microscopy were used to visualize the asymmetry of division and par2:GFP localization after the first cell division. Posterior is to the left.

Fig. 6 shows alterations in anterior-posterior patterning after treatment with concentramide. Figs. 6A-6C, *In situ* hybridization was used to show Pax2.1 expression in untreated (Fig. 6A) and concentramide-treated (Fig. 6B) 18-somite embryos. The expression patterns have been false-colored blue for untreated embryos and red for concentramide-treated embryos. Fig. 6C shows an overlay of the images from Figs. 6A and 6B. Arrowheads indicate areas of Pax2.1 expression at the midbrain-hindbrain boundary and in the otic placodes. The view is lateral, anterior to the left in Figs. 6A-6C. Fig. 6D, The distance between the anterior edge of the heart field, as defined by cmlc2 *in situ* staining, and the rostral extreme of the zebrafish embryo was measured in wild-type (WT), concentramide-treated (conc.), and *has* embryos at the 18-somite stage. Error bars represent standard error.

Fig. 7 shows the order of anterior and posterior heart field fusion. Dorsal views of cmlc2 expression at the16-somite (Figs. 7A-7C) and 18-somite (Figs. 7D-7F) stages. Expression patterns for wild-type (Fig. 7A and Fig. 7D), concentramide-treated (Fig. 7B and Fig. 7E), and *has* (Fig. 7C and 7F) embryos are shown. Anterior is up.

Fig. 8 is a schematic representation of a model for chamber patterning in the zebrafish heart. Normally, the bilateral primordia of the heart field converge and fuse first at the posterior end, followed by the anterior end to form a cone. The cone then rotates to orient atrial precursors toward the anterior and ventricular precursors toward the posterior in an extended heart tube. In concentramide-treated and *has* mutant embryos, the fusion order of the ends of the heart field is reversed, proceeding from the anterior to the posterior end. Rotation of the cone is blocked, preventing formation of the heart tube and causing the concentric heart chamber phenotype. Presumptive atrial precursor cells are colored red, ventricular precursor cells are colored blue. Views are dorsal; anterior is up.

## Detailed Description

The invention provides methods of diagnosing, preventing, and treating diseases and conditions associated with PKCλ, such as diseases or conditions of the heart (also

see below), and screening methods for identifying compounds that can be used to treat or to prevent such diseases and conditions. In particular, we have identified a small molecule, concentramide, and a genetic mutation, *heart-and-soul* (*has*), which disrupt the earliest heart. Both cause the ventricle to form within the atrium. We show here that the *has* gene encodes an atypical Protein Kinase C, Protein Kinase C $\lambda$ (PKC$\lambda$). The *has* mutation results in the disruption of epithelial cell-cell interactions in a broad range of tissues. Concentramide does not disrupt epithelial cell interactions but, rather, shifts the converging heart field of developing embryos rostrally. What is shared between the effects of concentramide and *has* is a reversal of the order of fusion of the anterior and posterior ends of the heart field.

The diagnostic methods of the invention thus involve detection of mutations in genes encoding PKC$\lambda$ proteins, while the compound identification methods involve screening for compounds that affect the phenotype of organisms having mutations in genes encoding PKC$\lambda$ or other models of appropriate diseases and conditions. The compound identification methods can also involve screening of candidate compounds in the presence of concentramide, using organisms with or without a PKC$\lambda$ mutation (e.g., the *has* mutation). Compounds identified in this manner, as well as PKC$\lambda$ genes and proteins themselves, can be used in methods to treat or prevent diseases and conditions associated with PKC$\lambda$. Compounds, antisense molecules, and antibodies that are found to inhibit PKC$\lambda$ function can also be used to prevent or treat cancer.

The invention also provides animal model systems (e.g., zebrafish having mutations (e.g., the *heart and soul* mutation) in PKC$\lambda$ genes, or mice (or other animals) having such mutations) that can be used in the screening methods mentioned above, as well as the PKC$\lambda$ protein, and genes encoding this protein. Also included in the invention are genes encoding mutant zebrafish PKC$\lambda$ proteins (e.g., genes having the *heart and soul* mutation) and proteins encoded by these genes. Antibodies that specifically bind to these proteins (wild type or mutant) are also included in the invention.

The diagnostic, screening, and therapeutic methods of the invention, as well as the animal model systems, proteins, and genes of the invention, are described further, as follows, after a brief description of diseases and conditions associated with PKC$\lambda$, which can be diagnosed, prevented, or treated according to the invention.

PKCλ-Associated Diseases or Conditions

Abnormalities in PKCλ genes or proteins can be associated with any of a wide variety of diseases or conditions, all of which can thus be diagnosed, prevented, or treated using the methods of the invention. For example, as discussed above, the *heart and soul* mutation in zebrafish is characterized by abnormal heart growth and development. Thus, detection of abnormalities in PKCλ genes or their expression can be used in methods to diagnose, or to monitor the treatment or development of, diseases or conditions of heart. In addition, compounds that are identified in the screening methods described herein, as well as PKCλ nucleic acid molecules, proteins, and antibodies themselves, can be used in methods to prevent or treat such diseases or conditions.

Specific examples of diseases or conditions of the heart that can be diagnosed, prevented, or treated according to the invention include congenital defects that result in heart malformation. These include congenital defects, such as Ebstein anomaly, which results in abnormalities of the tricuspid valve, as well as isomerism defects, which are characterized by a wide variety of abnormalities in the asymmetrical arrangement of particular organs, such as the heart, organs of the digestive tract, and the spleen, that normally occurs during development.

In right isomerism sequence, for example, which is also known as asplenia syndrome, Ivemark syndrome, and right atrial isomerism, the right side structures of the heart are duplicated on the left side of the heart, and the spleen is absent. This condition can lead to very complex and severe heart defects, such as atrioventricular septal defect (AVSD). In contrast, in left isomerism sequence, which is also known as polysplenia syndrome, the left side heart structures are duplicated and multiple small spleens may be present. This condition can lead to heart defects as well, such as heart block, which results in a slow heart beat, atrial septal defect, which is characterized by a hole between the top two heart chambers, and AVSD. With both types of isomerisms, twisting of the bowel or intestinal obstruction may result, due to the incorrect positioning of the intestines. Related defects may occur in other organs, such as the kidney.

Other diseases and conditions related to PKCλ that can be diagnosed, prevented, or treated according to the invention include those that are characterized by abnormalities in tight junctions. As is noted above, we have found that abnormalities in PKCλ (caused, e.g., by the *has* mutation) can lead to defects in epithelial cell-cell interactions. This is due to abnormalities in the formation of tight junctions, which play

critical roles in the sealing of spaces between the individual epithelial or endothelial cells that make up sheets of these cells that line the cavities of the body (e.g., the gastrointestinal tract, blood vessels, the respiratory tract, and the urinary tract), as well as enclose and protect certain organs (e.g., the brain). These sheets of cells function as selective permeability barriers, and alteration of the permeability of these barriers, due to, e.g., a PKCλ defect, can lead to any of a number of diseases or conditions that are well known in the art. For example, increased permeability of the lining of the gastrointestinal tract can lead to Crohn's disease, acute gastroenteritis, and diarrhea. Also, defects in tight junctions can interfere with the critical functions of the blood/brain barrier or the blood/retina barrier. As an additional example, vascular permeability defects in diabetic patients can lead to conditions such as diabetic retinopathy. Additional diseases and conditions that can be diagnosed, prevented, or treated, according to the invention, include those that are associated with abnormalities in epithelial cell polarity, such as polycystic kidney disease (e.g., autosomal dominant polycystic kidney disease). Also, because we have found that abnormalities in PKCλ lead to defects in cell growth control, a role for PKCλ in cancer is indicated. Compounds that are found to modulate PKCλ activity, thus, can be used in the prevention and treatment of cancer, such as, for example, carcinomas (e.g., renal cell carcinoma), which are cancers derived from epithelial cells.

Diagnostic Methods

Nucleic acid molecules encoding PKCλ proteins, as well as polypeptides encoded by these nucleic acid molecules and antibodies specific for these polypeptides, can be used in methods to diagnose or to monitor diseases and conditions involving mutations in, or inappropriate expression of, genes encoding this protein.

The diagnostic methods of the invention can be used, for example, with patients that have a disease or condition associated with PKCλ, in an effort to determine its etiology and, thus, to facilitate selection of an appropriate course of treatment. The diagnostic methods can also be used with patients who have not yet developed, but who are at risk of developing, such a disease or condition, or with patients that are at an early stage of developing such a disease or condition. Also, the diagnostic methods of the invention can be used in prenatal genetic screening, for example, to identify parents who may be carriers of a recessive mutation in a gene encoding a PKCλ protein. The

methods of the invention can be used to diagnose (or to treat) the disorders described herein in any mammal, for example, in humans, domestic pets, or livestock.

Abnormalities in PKCλ that can be detected using the diagnostic methods of the invention include those characterized by, for example, (i) a gene encoding a PKCλ protein containing a mutation that results in the production of an abnormal PKCλ protein, (ii) an abnormal PKCλ polypeptide itself (e.g., a truncated protein), and (iii) a mutation in a PKCλ gene that results in production of an abnormal amount of this protein. Detection of such abnormalities can be used to diagnose human diseases or conditions related to PKCλ, such as those affecting the heart. Exemplary of the mutations in PKCλ genes is the *heart and soul* mutation, which is described further below.

A mutation in a PKCλ gene can be detected in any tissue of a subject, even one in which this protein is not expressed. Because of the possibly limited number of tissues in which these proteins may be expressed, for limited time periods, and because of the possible undesirability of sampling such tissues (e.g., heart tissue) for assays, it may be preferable to detect mutant genes in other, more easily obtained sample types, such as in blood or amniotic fluid samples.

Detection of a mutation in a gene encoding a PKCλ protein can be carried out using any standard diagnostic technique. For example, a biological sample obtained from a patient can be analyzed for one or more mutations (e.g., a *heart and soul* mutation) in nucleic acid molecules encoding a PKCλ protein using a mismatch detection approach. Generally, this approach involves polymerase chain reaction (PCR) amplification of nucleic acid molecules from a patient sample, followed by identification of a mutation (i.e., a mismatch) by detection of altered hybridization, aberrant electrophoretic gel migration, binding, or cleavage mediated by mismatch binding proteins, or by direct nucleic acid molecule sequencing. Any of these techniques can be used to facilitate detection of a mutant gene encoding a PKCλ protein, and each is well known in the art. For instance, examples of these techniques are described by Orita et al. (Proc. Natl. Acad. Sci. U.S.A. 86:2766-2770, 1989) and Sheffield et al. (Proc. Natl. Acad. Sci. U.S.A. 86:232-236, 1989).

As noted above, in addition to facilitating diagnosis of an existing disease or condition, mutation detection assays also provide an opportunity to diagnose a predisposition to disease related to a mutation in a PKCλ gene before the onset of

symptoms. For example, a patient who is heterozygous for a gene encoding an abnormal PKCλ protein (or an abnormal amount thereof) that suppresses normal PKCλ biological activity or expression may show no clinical symptoms of a disease related to such proteins, and yet possess a higher than normal probability of developing such disease. Given such a diagnosis, a patient can take precautions to minimize exposure to adverse environmental factors, and can carefully monitor their medical condition, for example, through frequent physical examinations. As mentioned above, this type of diagnostic approach can also be used to detect a mutation in a gene encoding the PKCλ protein in prenatal screens.

While it may be preferable to carry out diagnostic methods for detecting a mutation in a PKCλ gene using genomic DNA from readily accessible tissues, as noted above, mRNA encoding this protein, or the protein itself, can also be assayed from tissue samples in which it is expressed. Expression levels of a gene encoding PKCλ in such a tissue sample from a patient can be determined by using any of a number of standard techniques that are well known in the art, including northern blot analysis and quantitative PCR (see, e.g., Ausubel et al., supra; PCR Technology: Principles and Applications for DNA Amplification, H.A. Ehrlich, Ed., Stockton Press, NY; Yap et al. Nucl. Acids. Res. 19:4294, 1991).

In another diagnostic approach of the invention, an immunoassay is used to detect or to monitor the level of a PKCλ protein in a biological sample. Polyclonal or monoclonal antibodies specific for the PKCλ protein can be used in any standard immunoassay format (e.g., ELISA, Western blot, or RIA; see, e.g., Ausubel et al., supra) to measure polypeptide the levels of PKCλ. These levels can be compared to levels of PKCλ in a sample from an unaffected individual. Detection of a decrease in production of PKCλ using this method, for example, may be indicative of a condition or a predisposition to a condition involving insufficient biological activity of the PKCλ protein.

Immunohistochemical techniques can also be utilized for detection of PKCλ protein in patient samples. For example, a tissue sample can be obtained from a patient, sectioned, and stained for the presence of PKCλ using an anti-PKCλ antibody and any standard detection system (e.g., one that includes a secondary antibody conjugated to an enzyme, such as horseradish peroxidase). General guidance regarding such techniques

can be found in, e.g., Bancroft et al., Theory and Practice of Histological Techniques, Churchill Livingstone, 1982, and Ausubel et al., supra.

Identification of Molecules that can be used to Treat or to Prevent Diseases or Conditions Associated with PKCλ

Identification of a mutation in the gene encoding PKCλ as resulting in a phenotype that results in abnormal heart growth and development facilitates the identification of molecules (e.g., small organic or inorganic molecules, antibodies, peptides, or nucleic acid molecules) that can be used to treat or to prevent diseases or conditions associated with PKCλ, as discussed above. The effects of candidate compounds on such diseases or conditions can be investigated using, for example, the zebrafish system. As is mentioned above, the zebrafish, *Danio rerio*, is a convenient organism to use in the genetic analysis of development. It has an accessible and transparent embryo, allowing direct observation of organ function from the earliest stages of development, has a short generation time, and is fecund. As discussed further below, zebrafish and other animals having a PKCλ mutation, such as the *heart and soul* mutation, which can be used in these methods, are also included in the invention.

In one example of the screening methods of the invention, a zebrafish having a mutation in a gene encoding the PKCλ protein (e.g., a zebrafish having the *heart and soul* mutation) is contacted with a candidate compound, and the effect of the compound on the development of a heart abnormality, or on the status of such an existing abnormality, is monitored relative to an untreated, identically mutant control. In a variation of this method, a zebrafish, with or without a mutation in the PKCλ gene (e.g., the *has* mutation), is contacted with a candidate compound in the presence of concentramide.

After a compound has been shown to have a desired effect in the zebrafish system, it can be tested in other models of heart disease, for example, in mice or other animals having a mutation in a gene encoding PKCλ. Alternatively, testing in such animal model systems can be carried out in the absence of zebrafish testing. Compounds of the invention can also be tested in animal models of cancer.

Cell culture-based assays can also be used in the identification of molecules that increase or decrease PKCλ levels or biological activity. According to one approach, candidate molecules are added at varying concentrations to the culture medium of cells

expressing PKCλ mRNA. PKCλ biological activity is then measured using standard techniques. The measurement of biological activity can include the measurement of PKCλ protein and nucleic acid molecule levels.

In general, novel drugs for the prevention or treatment of diseases related to mutations in genes encoding PKCλ can be identified from large libraries of natural products, synthetic (or semi-synthetic) extracts, and chemical libraries using methods that are well known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening methods of the invention and that dereplication, or the elimination of replicates or repeats of materials already known for their therapeutic activities for PKCλ, can be employed whenever possible.

Candidate compounds to be tested include purified (or substantially purified) molecules or one or more components of a mixture of compounds (e.g., an extract or supernatant obtained from cells; Ausubel et al., supra), and such compounds further include both naturally occurring or artificially derived chemicals and modifications of existing compounds. For example, candidate compounds can be polypeptides, synthesized organic or inorganic molecules, naturally occurring organic or inorganic molecules, nucleic acid molecules, and components thereof.

Numerous sources of naturally occurring candidate compounds are readily available to those skilled in the art. For example, naturally occurring compounds can be found in cell (including plant, fungal, prokaryotic, and animal) extracts, mammalian serum, growth medium in which mammalian cells have been cultured, protein expression libraries, or fermentation broths. In addition, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographic Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). Furthermore, libraries of natural compounds can be produced, if desired, according to methods that are known in the art, e.g., by standard extraction and fractionation.

Artificially derived candidate compounds are also readily available to those skilled in the art. Numerous methods are available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, for example, saccharide-, lipid-, peptide-, and nucleic acid molecule-based

compounds. In addition, synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemicals (Milwaukee, WI). Libraries of synthetic compounds can also be produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation. Furthermore, if desired, any library or compound can be readily modified using standard chemical, physical, or biochemical methods. The techniques of modern synthetic chemistry, including combinatorial chemistry, can also be used (reviewed in Schreiber, Bioorganic and Medicinal Chemistry 6:1172-1152, 1998; Schreiber, Science 287:1964-1969, 2000).

When a crude extract is found to have an effect on the development or persistence of a PKCλ-associated disease, further fractionation of the positive lead extract can be carried out to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having a desired activity. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives of these compounds. Methods of fractionation and purification of such heterogeneous extracts are well known in the art. If desired, compounds shown to be useful agents for treatment can be chemically modified according to methods known in the art.

In general, compounds that are found to activate PKCλ expression or activity may be used in the prevention or treatment of diseases or conditions of heart, such as those that are characterized by abnormal growth or development, or heart failure (also see above). Compounds that are found to modulate, e.g., block PKCλ expression or activity may be used to prevent or to treat cancer.

Animal Model Systems

The invention also provides animal model systems for use in carrying out the screening methods described above. Examples of these model systems include zebrafish and other animals, such as mice, that have a mutation (e.g., the *heart and soul* mutation) in a PKCλ gene. For example, a zebrafish model that can be used in the invention can include a mutation that results in a lack of PKCλ protein production or production of a truncated (e.g., by introduction of a stop codon) or otherwise altered PKCλ gene product.

As a specific example, a zebrafish having the *heart and soul* mutation can be used (see below).

Treatment or Prevention of PKCλ -Associated Diseases or Conditions

Compounds identified using the screening methods described above can be used to treat patients that have or are at risk of developing diseases or conditions of the heart or cancer. Nucleic acid molecules encoding the PKCλ protein, as well as these proteins themselves, can also be used in such methods. Treatment may be required only for a short period of time or may, in some form, be required throughout a patient's lifetime. Any continued need for treatment, however, can be determined using, for example, the diagnostic methods described above. In considering various therapies, it is to be understood that such therapies are, preferably, targeted to the affected or potentially affected organ (e.g., the heart). Such targeting can be achieved using standard methods.

Treatment or prevention of diseases resulting from a mutated PKCλ gene can be accomplished, for example, by modulating the function of a mutant PKCλ protein. Treatment can also be accomplished by delivering normal PKCλ protein to appropriate cells, altering the levels of normal or mutant PKCλ protein, replacing a mutant gene encoding a PKCλ protein with a normal gene encoding a PKCλ protein, or administering a normal gene encoding a PKCλ protein. It is also possible to correct the effects of a defect in a gene encoding a PKCλ protein by modifying the physiological pathway (e.g., a signal transduction pathway) in which a PKCλ protein participates.

In a patient diagnosed as being heterozygous for a gene encoding a mutant PKCλ protein, or as susceptible to such mutations or aberrant PKCλ expression (even if those mutations or expression patterns do not yet result in alterations in expression or biological activity of PKCλ), any of the therapies described herein can be administered before the occurrence of the disease phenotype. In particular, compounds shown to have an effect on the phenotype of mutants, or to modulate expression of PKCλ proteins, can be administered to patients diagnosed with potential or actual disease by any standard dosage and route of administration.

Any appropriate route of administration can be employed to administer a compound identified as described above, a PKCλ gene, or a PKCλ protein, according to the invention. For example, administration can be parenteral, intravenous, intra-arterial,

subcutaneous, intramuscular, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, by aerosol, by suppository, or oral.

A therapeutic compound of the invention can be administered within a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form. Administration can begin before or after the patient is symptomatic. Methods that are well known in the art for making formulations are found, for example, in Remington's Pharmaceutical Sciences (18$^{th}$ edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA. Therapeutic formulations can be in the form of liquid solutions or suspensions. Formulations for parenteral administration can contain, for example, excipients, sterile water, or saline; polyalkylene glycols, such as polyethylene glycol; oils of vegetable origin; or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers can be used to control the release of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. For oral administration, formulations can be in the form of tablets or capsules. Formulations for inhalation can contain excipients, for example, lactose, or can be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate, and deoxycholate, or can be oily solutions for administration in the form of nasal drops or as a gel. Alternatively, intranasal formulations can be in the form of powders or aerosols.

To replace a mutant protein with normal protein, or to add protein to cells that do not express a sufficient amount of PKCλ or normal PKCλ, it may be necessary to obtain large amounts of pure PKCλ protein from cell culture systems in which the protein is expressed (see, e.g., below). Delivery of the protein to the affected tissue can then be accomplished using appropriate packaging or administration systems.

Gene therapy is another therapeutic approach for preventing or ameliorating diseases caused by PKCλ gene defects. Nucleic acid molecules encoding wild type PKCλ protein can be delivered to cells that lack sufficient, normal PKCλ protein biological activity (e.g., cells carrying mutations (e.g., the *heart and soul* mutation) in PKCλ genes). The nucleic acid molecules must be delivered to those cells in a form in which they can be taken up by the cells and so that sufficient levels of protein, to provide effective PKCλ protein function, can be produced. Alternatively, for some PKCλ mutations, it may be possible to slow the progression of the resulting disease or to

modulate PKCλ protein activity by introducing another copy of a homologous gene bearing a second mutation in that gene, to alter the mutation, or to use another gene to block any negative effect.

Transducing viral (e.g., retroviral, adenoviral, and adeno-associated viral) vectors can be used for somatic cell gene therapy, especially because of their high efficiency of infection and stable integration and expression (see, e.g., Cayouette et al., Human Gene Therapy 8:423-430, 1997; Kido et al., Current Eye Research 15:833-844, 1996; Bloomer et al., Journal of Virology 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; and Miyoshi et al., Proc. Natl. Acad. Sci. U.S.A. 94:10319, 1997). For example, the full length PKCλ gene, or a portion thereof, can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for a target cell type of interest. Other viral vectors that can be used include, for example, a vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis et al., BioTechniques 6:608-614, 1988; Tolstoshev et al., Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotechnology 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Patent No. 5,399,346).

Non-viral approaches can also be employed for the introduction of therapeutic DNA into cells predicted to be subject to diseases involving the PKCλ protein. For example, a PKCλ nucleic acid molecule or an antisense nucleic acid molecule can be introduced into a cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990).

Gene transfer can also be achieved using non-viral means involving transfection *in vitro*. Such methods include the use of calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a patient can also be accomplished by transferring a normal PKCλ protein into a cultivatable cell type *ex vivo* (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue.

PKCλ cDNA expression for use in gene therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct PKCλ expression. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a PKCλ genomic clone is used as a therapeutic construct (such clones can be identified by hybridization with PKCλ cDNA, as described herein), regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Molecules for effecting antisense-based strategies can be employed to explore PKCλ protein gene function, as a basis for therapeutic drug design, as well as to treat PKCλ-associated diseases, such as cancer. These strategies are based on the principle that sequence-specific suppression of gene expression (via transcription or translation) can be achieved by intracellular hybridization between genomic DNA or mRNA and a complementary antisense species. The formation of a hybrid RNA duplex interferes with transcription of the target PKCλ-encoding genomic DNA molecule, or processing, transport, translation, or stability of the target PKCλ mRNA molecule.

Antisense strategies can be delivered by a variety of approaches. For example, antisense oligonucleotides or antisense RNA can be directly administered (e.g., by intravenous injection) to a subject in a form that allows uptake into cells. Alternatively, viral or plasmid vectors that encode antisense RNA (or antisense RNA fragments) can be introduced into a cell *in vivo* or *ex vivo*. Antisense effects can be induced by control (sense) sequences; however, the extent of phenotypic changes is highly variable.

Phenotypic effects induced by antisense molecules are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels.

PKCλ gene therapy can also be accomplished by direct administration of antisense PKCλ mRNA to a cell that is expected to be adversely affected by the expression of wild type or mutant PKCλ protein. The antisense PKCλ mRNA can be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an antisense PKCλ cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of antisense PKCλ mRNA to cells can be carried out by any of the methods for direct nucleic acid molecule administration described above.

An alternative strategy for inhibiting PKCλ protein function using gene therapy involves intracellular expression of an anti-PKCλ protein antibody or a portion of an anti-PKCλ protein antibody. For example, the gene (or gene fragment) encoding a monoclonal antibody that specifically binds to a PKCλ protein and inhibits its biological activity can be placed under the transcriptional control of a tissue-specific gene regulatory sequence.

Another therapeutic approach included in the invention involves administration of a recombinant PKCλ polypeptide, either directly to the site of a potential or actual disease-affected tissue (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of the PKCλ protein depends on a number of factors, including the size and health of the individual patient but, generally, between 0.1 mg and 100 mg, inclusive, is administered per day to an adult in any pharmaceutically acceptable formulation.

In addition to the therapeutic methods described herein, involving administration of PKCλ-modulating compounds, PKCλ proteins, or PKCλ nucleic acids to patients, the invention provides methods of culturing organs in the presence of such molecules. In particular, as is noted above, a PKCλ mutation is associated with abnormal heart growth and development. Thus, culturing heart tissue in the presence of these molecules can be used to promote its growth and development. This tissue can be that which is being prepared for transplant from, e.g., an allogeneic or xenogeneic donor, as well as synthetic tissue or organs.

Synthesis of PKCλ Proteins, Polypeptides, and Polypeptide Fragments

Those skilled in the art of molecular biology will understand that a wide variety of expression systems can be used to produce recombinant PKCλ proteins. As discussed further below, the precise host cell used is not critical to the invention. The PKCλ proteins can be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *S. cerevisiae*, insect cells, such as Sf9 cells, or mammalian cells, such as COS-1, NIH 3T3, or HeLa cells). These cells are commercially available from, for example, the American Type Culture Collection, Manassas, VA (see also Ausubel et al., supra). The method of transformation and the choice of expression vehicle (e.g., expression vector) will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al., supra, and expression vehicles can be chosen from those provided, e.g., in Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, Supp. 1987. Specific examples of expression systems that can be used in the invention are described further as follows.

For protein expression, eukaryotic or prokaryotic expression systems can be generated in which PKCλ gene sequences are introduced into a plasmid or other vector, which is then used to transform living cells. Constructs in which full-length PKCλ cDNAs, containing the entire open reading frame, inserted in the correct orientation into an expression plasmid, can be used for protein expression. Alternatively, portions of PKCλ gene sequences, including wild type or mutant PKCλ sequences, can be inserted. Prokaryotic and eukaryotic expression systems allow various important functional domains of PKCλ proteins to be recovered, if desired, as fusion proteins, and then used for binding, structural, and functional studies, and also for the generation of antibodies.

Typical expression vectors contain promoters that direct synthesis of large amounts of mRNA corresponding to a nucleic acid molecule that has been inserted into the vector. They can also include a eukaryotic or prokaryotic origin of replication, allowing for autonomous replication within a host cell, sequences that confer resistance to an otherwise toxic drug, thus allowing vector-containing cells to be selected in the presence of the drug, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable, long-term vectors can be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines can also be produced that

have the vector integrated into genomic DNA of the cells and, in this manner, the gene product can be produced in the cells on a continuous basis.

Expression of foreign molecules in bacteria, such as *Escherichia coli*, requires the insertion of a foreign nucleic acid molecule, e.g., a PKCλ nucleic acid molecule, into a bacterial expression vector. Such plasmid vectors include several elements required for the propagation of the plasmid in bacteria, and for expression of foreign DNA contained within the plasmid. Propagation of only plasmid-bearing bacteria is achieved by introducing, into the plasmid, a selectable marker-encoding gene that allows plasmid-bearing bacteria to grow in the presence of an otherwise toxic drug. The plasmid also contains a transcriptional promoter capable of directing synthesis of large amounts of mRNA from the foreign DNA. Such promoters can be, but are not necessarily, inducible promoters that initiate transcription upon induction by culture under appropriate conditions (e.g., in the presence of a drug that activates the promoter). The plasmid also, preferably, contains a polylinker to simplify insertion of the gene in the correct orientation within the vector.

Once an appropriate expression vector containing a PKCλ gene, or a fragment, fusion, or mutant thereof, is constructed, it can be introduced into an appropriate host cell using a transformation technique, such as, for example, calcium phosphate transfection, DEAE-dextran transfection, electroporation, microinjection, protoplast fusion, or liposome-mediated transfection. Host cells that can be transfected with the vectors of the invention can include, but are not limited to, *E. coli* or other bacteria, yeast, fungi, insect cells (using, for example, baculoviral vectors for expression), or cells derived from mice, humans, or other animals. Mammalian cells can also be used to express PKCλ proteins using a virus expression system (e.g., a vaccinia virus expression system) described, for example, in Ausubel et al., supra.

*In vitro* expression of PKCλ proteins, fusions, polypeptide fragments, or mutants encoded by cloned DNA can also be carried out using the T7 late-promoter expression system. This system depends on the regulated expression of T7 RNA polymerase, an enzyme encoded in the DNA of bacteriophage T7. The T7 RNA polymerase initiates transcription at a specific 23 base pair promoter sequence called the T7 late promoter. Copies of the T7 late promoter are located at several sites on the T7 genome, but none are present in *E. coli* chromosomal DNA. As a result, in T7-infected *E. coli*, T7 RNA polymerase catalyzes transcription of viral genes, but not *E. coli* genes. In this

expression system, recombinant *E. coli* cells are first engineered to carry the gene encoding T7 RNA polymerase next to the *lac* promoter. In the presence of IPTG, these cells transcribe the T7 polymerase gene at a high rate and synthesize abundant amounts of T7 RNA polymerase. These cells are then transformed with plasmid vectors that carry a copy of the T7 late promoter protein. When IPTG is added to the culture medium containing these transformed *E. coli* cells, large amounts of T7 RNA polymerase are produced. The polymerase then binds to the T7 late promoter on the plasmid expression vectors, catalyzing transcription of the inserted cDNA at a high rate. Since each *E. coli* cell contains many copies of the expression vector, large amounts of mRNA corresponding to the cloned cDNA can be produced in this system and the resulting protein can be radioactively labeled.

Plasmid vectors containing late promoters and the corresponding RNA polymerases from related bacteriophages, such as T3, T5, and SP6, can also be used for *in vitro* production of proteins from cloned DNA. *E. coli* can also be used for expression using an M13 phage, such as mGPI-2. Furthermore, vectors that contain phage lambda regulatory sequences, or vectors that direct the expression of fusion proteins, for example, a maltose-binding protein fusion protein or a glutathione-S-transferase fusion protein, also can be used for expression in *E. coli*.

Eukaryotic expression systems are useful for obtaining appropriate post-translational modification of expressed proteins. Transient transfection of a eukaryotic expression plasmid containing a PKCλ gene into a eukaryotic host cell allows the transient production of a PKCλ protein by the transfected host cell. PKCλ proteins can also be produced by a stably-transfected eukaryotic (e.g., mammalian) cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public (see, e.g., Pouwels et al., supra), as are methods for constructing lines including such cells (see, e.g., Ausubel et al., supra).

In one example, cDNA encoding a PKCλ protein, fusion, mutant, or polypeptide fragment is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, integration of the *heart and soul* protein-encoding gene, into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene.

Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al., supra. These methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. The most commonly used DHFR-containing expression vectors are pCVSEII-DHFR and pAdD26SV(A) (described, for example, in Ausubel et al., supra). The host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR- cells, ATCC Accession No. CRL 9096) are among those that are most preferred for DHFR selection of a stably transfected cell line or DHFR-mediated gene amplification.

Another preferred eukaryotic expression system is the baculovirus system using, for example, the vector pBacPAK9, which is available from Clontech (Palo Alto, CA). If desired, this system can be used in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan et al. (Molecular and Cellular Biology 5:3610-3616, 1985).

Once a recombinant protein is expressed, it can be isolated from the expressing cells by cell lysis followed by protein purification techniques, such as affinity chromatography. In this example, an anti-PKCλ antibody, which can be produced by the methods described herein, can be attached to a column and used to isolate the recombinant PKCλ. Lysis and fractionation of PKCλ-harboring cells prior to affinity chromatography can be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be purified further by, e.g., high performance liquid chromatography (HPLC; e.g., see Fisher, *Laboratory Techniques In Biochemistry and Molecular Biology*, Work and Burdon, Eds., Elsevier, 1980).

Polypeptides of the invention, particularly short PKCλ fragments and longer fragments of the N-terminus and C-terminus of PKCλ, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful PKCλ fragments or analogs, as described herein.

<u>PKCλ Protein Fragments</u>

Polypeptide fragments that include various portions of PKCλ proteins are useful in identifying the domains of PKCλ that are important for its biological activities. Methods for generating such fragments are well known in the art (see, for example,

Ausubel et al., supra), using the nucleotide sequences provided herein. For example, a PKCλ protein fragment can be generated by PCR amplifying a desired PKCλ nucleic acid molecule fragment using oligonucleotide primers designed based upon PKCλ nucleic acid sequences. Preferably, the oligonucleotide primers include unique restriction enzyme sites that facilitate insertion of the amplified fragment into the cloning site of an expression vector (e.g., a mammalian expression vector, see above). This vector can then be introduced into a cell (e.g., a mammalian cell; see above) by artifice, using any of the various techniques that are known in the art, such as those described herein, resulting in the production of a PKCλ protein fragment in the cell containing the expression vector. PKCλ protein fragments (e.g., chimeric fusion proteins) can also be used to raise antibodies specific for various regions of the PKCλ protein using, for example, the methods described below.

PKCλ Protein Antibodies

To prepare polyclonal antibodies, PKCλ proteins, fragments of PKCλ proteins, or fusion proteins containing defined portions of PKCλ proteins can be synthesized in, e.g., bacteria by expression of corresponding DNA sequences contained in a suitable cloning vehicle. Fusion proteins are commonly used as a source of antigen for producing antibodies. Two widely used expression systems for *E. coli* are *lacZ* fusions using the pUR series of vectors and *trpE* fusions using the pATH vectors. The proteins can be purified, coupled to a carrier protein, mixed with Freund's adjuvant to enhance stimulation of the antigenic response in an inoculated animal, and injected into rabbits or other laboratory animals. Alternatively, protein can be isolated from PKCλ-expressing cultured cells. Following booster injections at bi-weekly intervals, the rabbits or other laboratory animals are then bled and the sera isolated. The sera can be used directly or can be purified prior to use by various methods, including affinity chromatography employing reagents such as Protein A-Sepharose, antigen-Sepharose, and anti-mouse-Ig-Sepharose. The sera can then be used to probe protein extracts from PKCλ-expressing tissue fractionated by polyacrylamide gel electrophoresis to identify PKCλ proteins. Alternatively, synthetic peptides can be made that correspond to antigenic portions of the protein and used to inoculate the animals.

To generate peptide or full-length protein for use in making, for example, PKCλ-specific antibodies, a PKCλ coding sequence can be expressed as a C-terminal or N-terminal fusion with glutathione S-transferase (GST; Smith et al., Gene 67:31-40, 1988). The fusion protein can be purified on glutathione-Sepharose beads, eluted with glutathione, cleaved with a protease, such as thrombin or Factor-Xa (at the engineered cleavage site), and purified to the degree required to successfully immunize rabbits. Primary immunizations can be carried out with Freund's complete adjuvant and subsequent immunizations performed with Freund's incomplete adjuvant. Antibody titers can be monitored by Western blot and immunoprecipitation analyses using the protease-cleaved PKCλ fragment of the GST-PKCλ protein. Immune sera can be affinity purified using CNBr-Sepharose-coupled PKCλ. Antiserum specificity can be determined using a panel of unrelated GST fusion proteins.

Alternatively, monoclonal PKCλ antibodies can be produced by using, as an antigen, PKCλ isolated from PKCλ-expressing cultured cells or PKCλ protein isolated from tissues. The cell extracts, or recombinant protein extracts containing PKCλ, can, for example, be injected with Freund's adjuvant into mice. Several days after being injected, the mouse spleens can be removed, the tissues disaggregated, and the spleen cells suspended in phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes, some of which would be producing antibody of the appropriate specificity. These can then be fused with permanently growing myeloma partner cells, and the products of the fusion plated into a number of tissue culture wells in the presence of selective agents, such as hypoxanthine, aminopterine, and thymidine (HAT). The wells can then be screened by ELISA to identify those containing cells making antibodies capable of binding to PKCλ, polypeptide fragment, or mutant thereof. These cells can then be re-plated and, after a period of growth, the wells containing these cells can be screened again to identify antibody-producing cells. Several cloning procedures can be carried out until over 90% of the wells contain single clones that are positive for specific antibody production. From this procedure, a stable line of clones that produce the antibody can be established. The monoclonal antibody can then be purified by affinity chromatography using Protein A Sepharose and ion exchange chromatography, as well as variations and combinations of these techniques. Once produced, monoclonal antibodies are also tested for specific PKCλ recognition by Western blot or immunoprecipitation analysis (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et

al., European Journal of Immunology 6:511, 1976; Kohler et al., European Journal of Immunology 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, New York, NY, 1981; Ausubel et al., supra).

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic regions of PKCλ can be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides can be similarly affinity-purified on peptides conjugated to BSA, and specificity tested by ELISA and Western blotting using peptide conjugates, and by Western blotting and immunoprecipitation using PKCλ, for example, expressed as a GST fusion protein.

Antibodies of the invention can be produced using PKCλ amino acid sequences that do not reside within highly conserved regions, and that appear likely to be antigenic, as analyzed by criteria such as those provided by the Peptide Structure Program (Genetics Computer Group Sequence Analysis Package, Program Manual for the GCG Package, Version 7, 1991) using the algorithm of Jameson et al., CABIOS 4:181, 1988. These fragments can be generated by standard techniques, e.g., by PCR, and cloned into the pGEX expression vector. GST fusion proteins can be expressed in *E. coli* and purified using a glutathione-agarose affinity matrix (Ausubel et al., supra). To generate rabbit polyclonal antibodies, and to minimize the potential for obtaining antisera that is non-specific, or exhibits low-affinity binding to PKCλ, two or three fusions are generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in series, preferably including at least three booster injections.

In addition to intact monoclonal and polyclonal anti-PKCλ antibodies, the invention features various genetically engineered antibodies, humanized antibodies, and antibody fragments, including F(ab')2, Fab', Fab, Fv, and sFv fragments. Truncated versions of monoclonal antibodies, for example, can be produced by recombinant methods in which plasmids are generated that express the desired monoclonal antibody fragment(s) in a suitable host. Antibodies can be humanized by methods known in the art, e.g., monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA). Fully human antibodies, such as those expressed in transgenic animals, are also included in the invention (Green et al., Nature Genetics 7:13-21, 1994).

Ladner (U.S. Patent Nos. 4,946,778 and 4,704,692) describes methods for preparing single polypeptide chain antibodies. Ward et al., Nature 341:544-546, 1989, describes the preparation of heavy chain variable domains, which they term "single domain antibodies," and which have high antigen-binding affinities. McCafferty et al., Nature 348:552-554, 1990, shows that complete antibody V domains can be displayed on the surface of fd bacteriophage, that the phage bind specifically to antigen, and that rare phage (one in a million) can be isolated after affinity chromatography. Boss et al., U.S. Patent No. 4,816,397, describes various methods for producing immunoglobulins, and immunologically functional fragments thereof, that include at least the variable domains of the heavy and light chains in a single host cell. Cabilly et al., U.S. Patent No. 4,816,567, describes methods for preparing chimeric antibodies.

Use of PKCλ Antibodies

Antibodies to PKCλ can be used, as noted above, to detect PKCλ or to inhibit the biological activities of PKCλ. For example, a nucleic acid molecule encoding an antibody or portion of an antibody can be expressed within a cell to inhibit PKCλ function. In addition, the antibodies can be coupled to compounds, such as radionuclides and liposomes, for diagnostic or therapeutic uses. Antibodies that inhibit the activity of a PKCλ polypeptide described herein can also be useful in preventing or slowing the development of a disease caused by inappropriate expression of a wild type or mutant PKCλ gene.

Detection of PKCλ Gene Expression

As noted, the antibodies described above can be used to monitor PKCλ gene expression. *In situ* hybridization of RNA can be used to detect the expression of PKCλ genes. RNA *in situ* hybridization techniques rely upon the hybridization of a specifically labeled nucleic acid probe to the cellular RNA in individual cells or tissues. Therefore, RNA *in situ* hybridization is a powerful approach for studying tissue- and temporal-specific gene expression. In this method, oligonucleotides, cloned DNA fragments, or antisense RNA transcripts of cloned DNA fragments corresponding to unique portions of PKCλ genes are used to detect specific mRNA species, e.g., in the tissues of animals, such as mice, at various developmental stages. Other gene expression detection

techniques are known to those of skill in the art and can be employed for detection of PKCλ gene expression.

Identification of Additional PKCλ Genes

Standard techniques, such as the polymerase chain reaction (PCR) and DNA hybridization, can be used to clone PKCλ gene homologues in other species and PKCλ-related genes in humans. PKCλ-related genes and homologues can be readily identified using low-stringency DNA hybridization or low-stringency PCR with human PKCλ probes or primers. Degenerate primers encoding human PKCλ or human PKCλ-related amino acid sequences can be used to clone additional PKCλ-related genes and homologues by RT-PCR.

Construction of Transgenic Animals and Knockout Animals

Characterization of PKCλ genes provides information that allows PKCλ knockout animal models to be developed by homologous recombination. Preferably, a PKCλ knockout animal is a mammal, most preferably a mouse. Similarly, animal models of PKCλ overproduction can be generated by integrating one or more PKCλ sequences into the genome of an animal, according to standard transgenic techniques. Moreover, the effect of PKCλ mutations (e.g., dominant gene mutations) can be studied using transgenic mice carrying mutated PKCλ transgenes or by introducing such mutations into the endogenous PKCλ gene, using standard homologous recombination techniques.

A replacement-type targeting vector, which can be used to create a knockout model, can be constructed using an isogenic genomic clone, for example, from a mouse strain such as 129/Sv (Stratagene Inc., LaJolla, CA). The targeting vector can be introduced into a suitably derived line of embryonic stem (ES) cells by electroporation to generate ES cell lines that carry a profoundly truncated form of a PKCλ gene. To generate chimeric founder mice, the targeted cell lines are injected into a mouse blastula-stage embryo. Heterozygous offspring can be interbred to homozygosity. PKCλ knockout mice provide a tool for studying the role of PKCλ in embryonic development and in disease. Moreover, such mice provide the means, *in vivo*, for testing therapeutic compounds for amelioration of diseases or conditions involving PKCλ-dependent or a PKCλ-effected pathway.

Use of PKCλ as a Marker for Stem Cells of the Heart

As PKCλ is expressed in cells that give rise to the heart during the course of development, it can be used as a marker for stem cells of the heart. For example, PKCλ can be used to identify, sort, or target such stem cells. A pool of candidate cells, for example, can be analyzed for PKCλ expression, to facilitate the identification of heart stem cells, which, based on this identification can be separated from the pool. The isolated stem cells can be used for many purposes that are known to those of skill in this art. For example, the stem cells can be used in the production of new organs, in organ culture, or to fortify damaged or transplanted organs.

Experimental Results

*Concentramide specifically modulates a biological pathway involved in heart patterning*

Zebrafish embryos have recently been shown to be amenable to high-throughput screening to identify small molecules that perturb developmental processes (Peterson et al., Proc. Natl. Acad. Sci. U.S.A. 97:12965-12969, 2000). In one such screen, we exposed developing zebrafish embryos to small molecules from a large, diverse chemical library. Visual inspection of the transparent embryos was used to identify small molecules that affect the global patterning of the heart. One of these small molecules is a biaryl compound containing an acrylamide moiety that we call concentramide (Fig. 1A), originally identified as library number 32P6 (Peterson et al., supra).

Normally, by 24 hours post-fertilization (hpf) the heart tube assembles in the midline, with the atrium anterior to the ventricle and slightly displaced towards the left (Fig. 2A), and blood flow is driven from atrial to ventricular end, first by persistalsis and then by sequential chamber contractions. By 30 hpf, the chambers are clearly demarcated (Fig. 2B, using cardiac myosin light chain 2, cmlc2, to label both chambers) and express different genes, as shown in Fig. 2C (ventricle-specific myosin heavy chain and atrial-specific antibody S46).

Embryos exposed to concentramide develop compact hearts that do not sustain a circulation. It appears that both the atrium and ventricle form and beat in a coordinated manner in these fish, but that the ventricle forms in the center of the atrium, as shown in Figs. 2E and 2F. The result is a heart in which the atrium and ventricle form two concentric rings, the inner ring composed of the ventricle and the outer ring composed of

the atrium. From the dorsal view, the heart looks like a bullseye (Fig. 2D), and from the lateral view, it looks like an inverted mushroom, in which the ventricle forms the stalk of the mushroom and the atrium surrounds and covers the ventricle like a mushroom cap (Fig. 1B).

Several observations suggest that concentramide is a highly specific modulator of a particular molecular pathway critical to heart patterning. Concentramide is very potent, with an $ED_{50}$ of about 2 nM. More importantly, higher doses of concentramide do not appear to cause additional side effects. Concentramide causes virtually the same phenotype when used at a concentration of 6 μM as it does when used at a concentration of 6 nM, suggesting that it modulates a specific molecular target at least 1,000 times more potently than it modulates other proteins affecting visible developmental processes. The effect of concentramide on cardiovascular development does not appear to be a result of general cytotoxicity. Development of concentramide-treated embryos is not delayed relative to untreated siblings, and no increase in cell death is apparent. Concentramide also has no effect on the rate of proliferation of yeast or bromodeoxyuridine incorporation in mammalian cells. Given the potency of concentramide, its phenotypic reproducibility over a broad concentration range, and the rarity of the phenotype it produces (none of the >2000 other small molecules screened generates a similar phenotype), we conclude that concentramide is a specific modulator of a biological pathway responsible for heart patterning.

*A time window for concentramide effects*

One advantage of small molecules over genetic mutations in studying a developmental process is that small molecules allow the process to be modulated with much greater temporal control. Small molecules can be added or washed away at any time during development, whereas genetic mutations are generally present throughout development. This temporal control afforded by small molecules facilitates the identification of critical periods for developmental processes.

To identify the developmental stage at which concentramide disrupts heart patterning decisions, we added concentramide to the water of developing embryos at various times. As shown in Fig. 1C, embryos treated at any time prior to 14 hpf exhibit the concentric chamber morphology at 24 hpf, while embryos treated after 17 hpf exhibit wild-type heart morphology at 24 hpf. Repeating the experiment with more precise

staging revealed that concentramide must be present before the 14-somite stage (approximately 15 hpf) to induce the concentric chamber morphology. Therefore, a developmental event occurring at the 14-somite stage is critical for heart patterning and is disrupted by the small molecule concentramide.

*The hearts of concentramide-treated embryos phenocopy heart-and-soul mutants*

*Heart-and-soul* (*has*) is a mutation isolated in our large-scale genetic screen. The hearts of homozygous *has* mutant embryos are small. We find here that, like those of concentramide-treated embryos, the hearts of *has* mutant embryos have ventricular tissue within the atrium (Figs. 2G and 2H). They manifest radial sequential contractions of the atrium, then the ventricle. The *has* mutant embryos, however, also manifest defects in many tissues including the retina, kidney, gut, and brain. These defects are not present in concentramide-treated embryos. The brains of concentramide-treated embryos develop abnormally, but treating embryos between 9 and 14 hpf eliminates this brain defect, while preserving the concentric heart chamber phenotype (Fig. 1C). Therefore, the heart phenotypes of concentramide-treated and *has* mutant embryos are very similar, but concentramide-treated embryos appear to have fewer developmental defects elsewhere, and the cardiac specificity of the phenotype can be increased further by controlling the timing of concentramide treatment.

*Heart-and-soul encodes an atypical PKCλ*

Given the phenotypic similarities between hearts from *has* and concentramide-treated embryos, we reasoned that cloning the *has* gene might provide molecular insight about the process of heart patterning. Furthermore, cloning of *has* might allow us to determine whether *has* and concentramide influence heart patterning through similar or distinct mechanisms. We mapped *has* by linkage analysis with zebrafish SSR markers (Michelmore et al., Proc. Natl. Acad. Sci. U.S.A. 88:9828-9832, 1991; Knapik et al., Nat. Genet. 18:338-343, 1998; Shimoda et al., Genomics 58:219-232, 1999) and AFLP (Vos et al., Nucleic Acids Res. 23:4407-4414, 1995) to an interval flanked by markers z8451 and z11023 of approximately 1.1cM (Fig. 3A). These were used to initiate a walk using YACs and BACs, which proceeded by end-cloning, refined mapping, and ultimately sequencing. Genes identified as candidates for the mutation were assayed by *in situ* analysis and for cDNA polymorphism by RT-PCR of wild-type and mutant RNA

pools. The genes contained within the BACs are shown in Table 1. The gene assignments are based on BLASTX alignments.

**Table 1. Candidate genes identified within the *heart-and-soul* interval**

| BAC address | identified genes (GenBank accession#) |
|---|---|
| 109f10/122n17 | KIAA0670 protein/acinus (NP_055792) |
| | membane-type 1 metalloproteinase precursor (AAD13803) |
| | adaptin, gamma (NP_001119) |
| | KIAA1416 protein, novel Helicase C-terminal domain and SNF2 N-termina domains containing protein, similar to KIAA0308 (CAB57836) |
| | ZPC domain containing protein 2 (AAD38907) |
| | zinc finger protein sal (AAB51127) |
| | cerebellin 1 precursor (NP_004343) |
| | RING finger protein (AAB05873) |
| 152p21 | unknown (NP_056541) |
| 89i15 | precerebellin-like protein (AAF04305) |
| 23c14 | PKCλ |
| | transforming protein sno-N - chicken (I51298) |
| 53c17 | no genes detected by BLASTX (mostly repetitive) |

By sequencing PKCλ from wild type and mutant embryos, we confirmed that both *has* alleles harbor mutations in the PKCλ coding sequence. The mutation in the m567 allele causes a premature stop codon after amino acid 518, and the mutation in the m129 allele causes a premature stop codon after amino acid 514 (Fig. 3A). We determined the complete genomic structure of the zebrafish PKCλ gene by shotgun sequencing of BAC 23c14. It is comprised of 18 exons spanning approximately 45 kb. We find PKCλ mRNA to be expressed in a broad range of tissues.

The C-terminal truncation of PKCλ does not appear to destabilize the protein, as truncated protein is detected by western blot analysis of mutant embryos (Fig. 3B). However, truncation might be predicted to eliminate a domain essential for PKCλ function, given that C-terminal truncation of PKCα or PKCβ renders these related kinases catalytically inactive (Riedel et al., J. Cell. Biochem. 52:320-329, 1993; Riedel et al., Mol. Cell. Biol. 13:4728-4735, 1993). In order to confirm the role of PKCλ mutation in the phenotype, we injected antisense morpholino oligomers complementary to the PKCλ translational start site. These injections phenocopy the mutation entirely. The injected embryos are indistinguishable at the gross morphological level from the

genetic mutants (Fig. 3C), supporting the idea that loss of the C-terminal 70 amino acids is sufficient to eliminate gene function.

*The integrity of epithelia is affected by PKCλ mutation, but not by treatment with concentramide*

PKCλ belongs to the large PKC family of kinases and, with PKCζ, is classified as an 'atypical' PKC (Mellor et al., Biochem. J. 332:281-292, 1998). The presumptive ortholog of PKCλ in *C. elegans*, PKC-3, colocalizes with Par3 and Par6 at the anterior pole of the one-cell embryo (Tabuse et al., supra; Hung et al., Development 126:127-135, 1999). PKC-3 is necessary for establishment of embryonic polarity, and inactivation of PKC-3 leads to mislocalization of the Par genes and a symmetrical first cell division. *Drosophila* possesses only one atypical PKC (DaPKC), which also associates with a Par3-like protein (Bazooka) and is implicated in control of cell polarity (Wordarz et al., supra). DaPKC mutants exhibit disordered epithelial layering, irregular cell shapes, and loss of epithelial cell polarity, believed to be due to defects in cell adhesion. In vertebrate cells, PKCλ and PKCζ both localize to epithelial tight junctions and associate with a Par3-like protein (ASIP) (Joberty et al., Nat. Cell Biol. 2:531-539, 2000; Suzuki et al., J. Cell Biol. 152:1183-1196, 2001; Lin et al., Nat. Cell Biol. 2:540-547, 2000; Izumi et al., J. Cell Biol. 143:95-106, 1998). We therefore examined whether the *has* mutation and concentramide treatment perturb epithelial patterning and tight junctions, focusing upon the retina and the kidney.

The neural retina arises from an epithelial sheet that is bordered by the lens on the basal surface and by a second epithelial sheet (the retinal pigmented epithelium, RPE) on the apical surface (Schmitt et al., J. Comp. Neurol. 344:532-542, 1994). Prior to cell differentiation, the nuclei of the neuroepithelial cells migrate between the apical and basal surfaces of the epithelium. During M-phase, cell nuclei localize to the apical surface, adjacent to the neighboring RPE (Sauer, J. Comp. Neurol. 62:377-405, 1935). Beginning at about 30 hpf, these neuroepithelial cells exit the cell cycle and differentiate into one of seven distinct cell types (Altshuler et al., "Specification of Cell Type in the Vertebrate Retina," *In* Development of the Visual System, Lam et al. (Eds.), The MIT Press, Cambridge, MA 37-58, 1991; Dowling, "The Retina," Belknap Press, Cambridge, MA, 1987). Each cell type then migrates to a specific layer in the retina, resulting in a highly organized, laminar pattern (see Fig. 4A).

The *has* mutation causes disruption of the layering of the neural retina and patchy loss of the RPE (Fig. 4E). These defects resemble those noted previously in zebrafish bearing the mutations *oko meduzy* (*ome*) and *mosaic eyes* (*moe*) (Jensen et al., Development 128:95-105, 2001; Malicki et al., Development 126:1235-1246, 1999). In *has* mutants, the severity of laminar disruption correlates with the position and degree of RPE discontinuity, suggesting that the RPE epithelial defect causes or exacerbates that of the neural retina. This would be concordant with the evidence that a normal RPE is critical to lamination (Raymond et al., Curr. Biol. 5:1286-1295, 1995; Vollmer et al., Neurosci. Lett. 48:191-196, 1984) and the fact that the retinal epithelium of *has* mutants manifests at least one attribute of proper apical-basal polarity in that the majority of the mitotic nuclei localize correctly to the apical surface of the neuroepithelium (Figs. 4B, 4D, and 4F; 89% of M-phase nuclei from *has* embryos localize to the apical surface versus 97% of nuclei from wild-type embryos). As a marker of tight junctions, we examined immunoreactive zonula occludens (ZO-1), an integral tight junction protein, and find it to be mislocalized (Figs. 4G and 4H). Therefore, loss of adhesion between RPE cells may be a cause of retinal mispatterning in *has* mutants. Notably, retinas from concentramide-treated embryos do not exhibit defects in cell polarity (Fig. 4D), RPE continuity, or lamination (Fig. 4C)

The developing kidney is another structure composed of highly polarized epithelial cells. We examined the distribution of apical and basolateral proteins in the kidneys of wild-type, *has*, and concentramide-treated embryos. As in the retina, cell polarity appeared to be largely conserved in *has* kidneys (Figs. 5A-5C). The *has* kidneys did, however, exhibit irregularities in the shapes of epithelial cells and occasional gaps between cells, consistent with a defect in epithelial cell adhesion. We did not observe these defects in embryos exposed to concentramide.

Given the differences between *has* and concentramide-treated embryos with regard to epithelial sheet integrity in the retina and the kidney, it is unlikely that concentramide functions through the same mechanism as the *has* mutation, namely the inactivation of PKCλ. To examine this further, we tested the effect of concentramide on early development of the *C. elegans* embryo. In *C. elegans*, inactivation of the PKCλ ortholog PKC-3 via RNA interference (RNAi) results in the loss of polarized localization of the Par proteins and loss of asymmetry during the first cell division (Tabuse et al., supra). Embryos treated with high concentrations of concentramide retain

proper localization of Par2 to the posterior pole and undergo a normally asymmetric first cell division (Figs. 5D and 5E). Treated embryos exhibit cytokinetic defects and fail to complete development, suggesting that the absence of an asymmetry defect is not due to problems with compound penetration. Therefore, although concentramide treatment and PKCλ inactivation both result in similar heart patterning phenotypes, concentramide does not appear to inactivate zebrafish PKCλ or its nematode ortholog.

*The molecular target of concentramide is involved in AP patterning*

If the molecular target of concentramide does not affect the continuity of epithelial sheets as PKCλ does, by what sort of process might it influence heart patterning? Treatment with concentramide appears to affect the relative positions of several anatomical structures along the anterior-posterior (AP) axis. For example, the distance between Pax2.1-expressing cells in the eyes and at the midbrain/hindbrain boundary is reduced in concentramide-treated embryos (Figs. 6A-6C). Perhaps more significantly, the cardiac myosin light chain 2 (cmlc2)-expressing cells of the heart field are shifted rostrally in concentramide-treated embryos at the 18-somite stage (Fig. 6D). The distance between the anterior edge of the cmlc2-expressing field and the anterior extreme of the embryo is about 40 percent greater in wild-type embryos (3.1 +/- 0.2 arbitrary units, n=8) than in concentramide-treated embryos (2.2 +/- 0.3 arbitrary units, n=12). The position of the heart field in *has* mutants (3.1 +/- 0.3 arbitrary units, n=12) does not differ significantly from the wild-type position. Therefore, the molecular target of concentramide appears to play a role in AP patterning.

*PKCλ and the target of concentramide both influence the fusion order of heart primordia*

PKCλ and the molecular target of concentramide appear to act via distinct cellular mechanisms, but modulation of either results in a very similar change in the patterning of the heart. To identify the commonalties between the two mechanisms that allow such similar mispatterning of the heart, we took advantage of the temporal control with which small molecules can modulate biological processes. As described above, we determined that embryos must be treated with concentramide at or prior to the 14-somite stage to cause formation of the ventricle within the atrium. From this observation, we conclude that a critical heart patterning process is initiated shortly after the 14-somite

stage, and perturbation of this process results in the concentric chamber phenotype observed in both *has* and concentramide-treated embryos. This allowed us to focus our search for commonalties between *has* and concentramide-treated embryos to this critical time period.

The generation of the primitive heart tube is accomplished by midline coalescence of the bilateral cardiac primordial sheets. In the zebrafish, this coalescence first generates a single midline cone, with its base on the yolk (Fishman et al., supra; Yelon et al., Dev. Biol. 214:23-37, 1999). Subsequently, the cone tilts to assume a midline A-P orientation with the pre-ventricular end posterior, later to swing anteriorly as yolk is resorbed.

We find that normally the generation of the midline cone does not occur uniformly around the cone's circumference, but rather progresses from posterior to anterior, with posterior regions merging at the 16-somite stage and anterior at the 18-somite stage. This step is perturbed by both concentramide and the *has* mutation. In both *has* mutant embryos and concentramide-treated embryos, there is a failure to merge the posterior ends (Figs. 7A-7C). Even by the 18-somite stage, when the anterior ends of the primordia begin to fuse normally, the posterior ends remain separated in the *has* and concentramide-treated embryos (Figs. 7D-7F). Eventually, the posterior ends do fuse in *has* and concentramide-treated embryos, just before emergence of the concentric chambered heart. Thus, a critical patterning decision occurs at about the 16-somite stage that regulates the fusion order of the anterior and posterior ends of the heart field. This process can be blocked either by inactivation of PKCλ or by modulating the target of concentramide.

Thus, in summary, we have defined a key step in heart formation by its perturbation with a small molecule and a mutation. This step involves the proper alignment of the two cardiac chambers, just as the primitive heart tube assembles. Two perturbants --the small molecule concentramide and the *has* mutation-- both elicit a previously undescribed chamber malalignment, in which the ventricle forms inside of the atrium. This means that establishment of the cardiocyte cell fates is largely accomplished, but the higher order assembly of chamber structure is disrupted.

Experimental Methods

*Small molecule treatment*

Zebrafish were maintained at 28.5°C as described (Westerfield, "The Zebrafish Book, Guide for the Laboratory Use of Zebrafish (*Danio rerio*)," Univ. of Oregon Press, Eugene 1995). Unless specified otherwise, embryos were treated prior to gastrulation by adding concentramide to the water at a final concentration of 34 nM from a 34 μM stock solution in DMSO.

*Whole-mount in situ hybridization and immunohistochemistry*

Digoxigenin-labeled antisense RNA probes were generated by *in vitro* transcription for cmlc2 (Yelon et al., supra), vmhc (Yelon et al., supra), and pax2.1 (Krauss et al., Development 113:1193-1206, 1991). *In situ* hybridization was carried out as described (Oxtoby et al., Nucleic Acids Res. 21:1087-1095, 1993). For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (S46 and 3G8) or 80% methanol, 20% dimethyl sulfoxide (α-ZO-1), permeablized in acetone for 30 minutes at -20°C (3G8), blocked with 5% fetal bovine serum, and incubated with the antibodies S46, 3G8 (Vize et al., Dev. Biol. 171:531-540, 1995), or α-ZO-1. An anti-mouse-horseradish peroxidase conjugate was used as secondary antibody for S46 and 3G8, and an Alexa 488-labeled anti-mouse secondary antibody was used for α-ZO-1 staining.

*Histology*

Fixed embryos were dehydrated, embedded in plastic (JB-4, Polysciences, Inc.), and sectioned at 2-7 μm. Retinal sections were stained with hematoxylin-eosin or dapi.

*Cloning of has*

Embryos were separated into mutant and wild-type pools based on phenotypic analysis. Genomic DNA was isolated from individual embryos by incubation in DNA isolation buffer overnight at 50°C (DNA isolation buffer: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.3% Tween-20; 0.3% Nonidet P40; 0.5 mg/ml proteinase K). Proteinase K was inactivated prior to PCR setup by heating samples to 98°C for 10 minutes. PCR

reactions were performed using diluted genomic DNA as described (Knapik et al., Development 123:451-460, 1996).

RNA was isolated (RNeasy columns, Qiagen) from pools of wild-type and mutant embryos to generate cDNA for RT-PCR analysis (SMART RACE cDNA amplification kit, Clontech). Fragments were then subcloned into PCRII-TOPO (Invitrogen). PCR primers were synthesized based on sequence from an EST for PKCλ (fc69h04, GenBank accession# AI883774) and genomic sequence (Genome Systems, BAC clone address 23c14), and used to sequence the entire PKCλ coding region and 3'UTR.

Genomic clones were isolated by PCR analysis of DNA pools from BAC (Genome Systems) and YAC (Research Genetics) libraries using primer sets for the linked markers z11023 and z8451. YAC end sequence was determined as described (Zhong et al., Genomics 48:136-138, 1998). BAC ends were sequenced directly using SP6 and T7 primers, and BACs 53c17, 89i15, and 152p21 were subcloned by shotgun cloning of partial AluI digested fragments into pBluescript. For the complete sequencing of BACs, a hydroshear was used to produce fragments of 2-3kb in length. These fragments were then blunt-end ligated into pGEM5 (Promega) and sequenced using an ABI3700 to generate approximately five-fold coverage. The sequence was assembled using the Phred/Phrap/Consed programs (Gordon et al., Genome Res. 8:195-202, 1998; Ewing et al., Genome Res. 8:186-194, 1998; Ewing et al., Genome Res. 8:175-185, 1998).

*Western blotting*

Groups of 25 embryos were lysed in 0.5% Triton X100 in phosphate-buffered saline. Lysates were clarified by centrifugation and separted by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting was performed using an α-PKCλ rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.).

*Morpholino injection*

An antisense morpholino oligonucleotide of sequence 5'-CTGTCCCGCAGCGTGGGCATTATGG-3' (GeneTools, LLC) was dissolved at a concentration of 100 μM in 1X Danieau's buffer (5 mM Hepes pH 7.6, 58 mM NaCl, 0.7 mM KCl, 0.6 mM $Ca(NO_3)_2$, 0.4 mM $MgSO_4$). One nL of this solution or 1X

Danieau's buffer was injected into each 1-4 cell embryo before allowing the embryos to develop at 28.5°C.

*C. elegans development*

*C. elegans* strain KK871 (par-2::GFP) was maintained at 25°C. For each sample, 10-15 adult worms were soaked in 80 μL M9 medium containing 34 μM concentramide, 0.25% dimethyl sulfoxide for 30-60 minutes. Worms were then cut open with a scalpel, and embryos were mounted on 2% agarose pads with coverslips. Embryos were allowed to develop at 25°C before being photographed live.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it is to be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and can be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

Claims

1. A method of determining whether a test subject has, or is at risk of developing, a disease or condition related to Protein Kinase C λ, said method comprising analyzing a nucleic acid molecule of a sample from the test subject to determine whether the test subject has a mutation in a gene encoding said Protein Kinase C λ, wherein the presence of a mutation indicates that said test subject has, or is at risk of developing, a disease or condition related to Protein Kinase C λ.

2. The method of claim 1, wherein said test subject is a mammal.

3. The method of claim 1, wherein said test subject is a human.

4. The method of claim 1, wherein said disease or condition is a disease or condition of the heart or cancer.

5. The method of claim 1, wherein said disease or condition is associated with epithelial-epithelial cell interactions or epithelial cell polarity.

6. The method of claim 1, wherein said mutation results in a carboxyl terminal truncation of Protein Kinase C λ.

7. The method of claim 1, wherein said mutation is the *heart and soul* mutation.

8. A method for identifying a compound that can be used to treat or to prevent a disease or condition of associated with Protein Kinase C λ, said method comprising contacting an organism comprising a mutation in a gene encoding Protein Kinase C λ and having a phenotype characteristic of a disease or condition associated with Protein Kinase C λ with said compound, and determining the effect of said compound on said phenotype, wherein detection of an improvement in said phenotype indicates the identification of a compound that can be used to treat or to prevent said disease or condition.

9. The method of claim 8, wherein said disease or condition associated with Protein Kinase C λ is heart disease.

10. The method of claim 8, wherein said organism is a zebrafish.

11. The method of claim 8, wherein said mutation results in a carboxyl terminal truncation of Protein Kinase C λ.

12. A method of treating or preventing a disease or condition associated with Protein Kinase C λ in a patient, said method comprising administering to said patient a compound identified using the method of claim 8.

13. The method of claim 12, wherein said disease or condition is of the heart.

14. The method of claim 12, wherein said patient has a mutation that results in a carboxyl terminal truncation of Protein Kinase C λ.

15. A method of treating or preventing a disease or condition associated with Protein Kinase C λ in a patient, said method comprising administering to said patient a functional Protein Kinase C λ protein or an expression vector comprising a nucleic acid molecule encoding said protein.

16. A method of treating or preventing a disease or condition associated with Protein Kinase C λ in a patient, said method comprising administering to said patient a compound or molecule that alters the activity or expression of Protein Kinase C λ in said patient.

17. A substantially pure zebrafish Protein Kinase C λ polypeptide.

18. The polypeptide of claim 17, wherein said polypeptide comprises an amino acid sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2.

19. The polypeptide of claim 17, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:2.

20. A substantially pure polypeptide comprising the sequence of SEQ ID NO:2 and variants thereof comprising sequences that are at least 95% identical to that of SEQ ID NO:2, and which have Protein Kinase C λ activity.

21. An isolated nucleic acid molecule comprising a sequence encoding a zebrafish Protein Kinase C λ polypeptide.

22. The nucleic acid molecule of claim 21, wherein said nucleic acid molecule encodes a polypeptide comprising an amino sequence that is substantially identical to the amino acid sequence of SEQ ID NO:2.

23. The nucleic acid molecule of claim 21, wherein said nucleic acid molecule encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2.

24. An isolated nucleic acid molecule that specifically hybridizes under high stringency conditions to the complement of the sequence set forth in SEQ ID NO:1, wherein said nucleic acid molecule encodes a protein that has Protein Kinase C λ activity.

25. A vector comprising the nucleic acid molecule of claim 21.

26. A cell comprising the vector of claim 25.

27. A non-human animal having a knockout mutation in one or both alleles encoding a Protein Kinase C λ polypeptide.

28. A cell from the non-human knockout animal of claim 27.

29. A non-human transgenic animal comprising a nucleic acid molecule encoding a mutant Protein Kinase C λ polypeptide.

30. The non-human transgenic animal of claim 29, wherein the non-human transgenic animal is a zebrafish.

31. The non-human transgenic animal of claim 29, wherein the non-human transgenic animal comprises the *heart and soul* mutation.

32. An antibody that specifically binds to a Protein Kinase C $\lambda$ polypeptide.

33. A method of modulating the activity of a Protein Kinase C $\lambda$ polypeptide in a patient, said method comprising administering to the patient an RNA that stimulates or inhibits this activity.

# Fig. 1

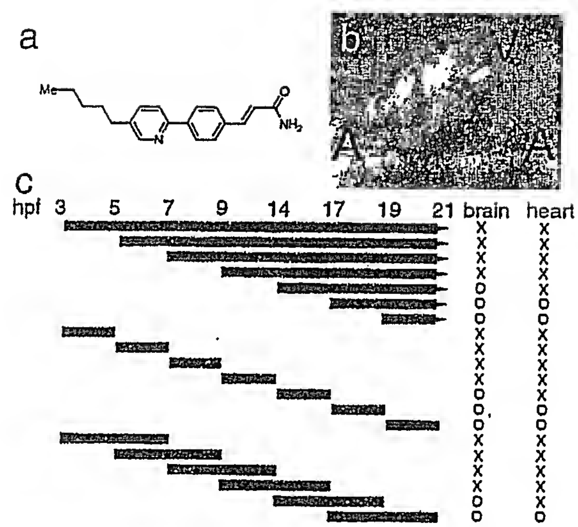


Fig. 2

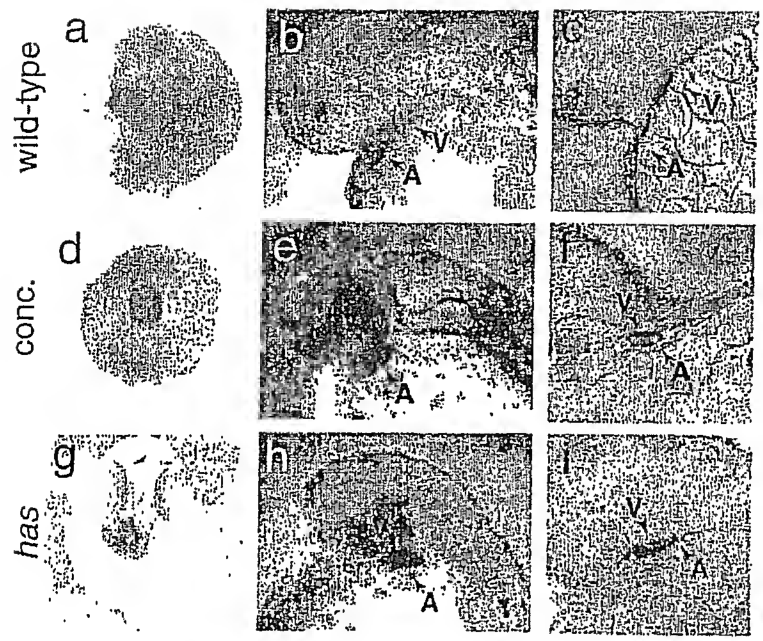


Fig. 3

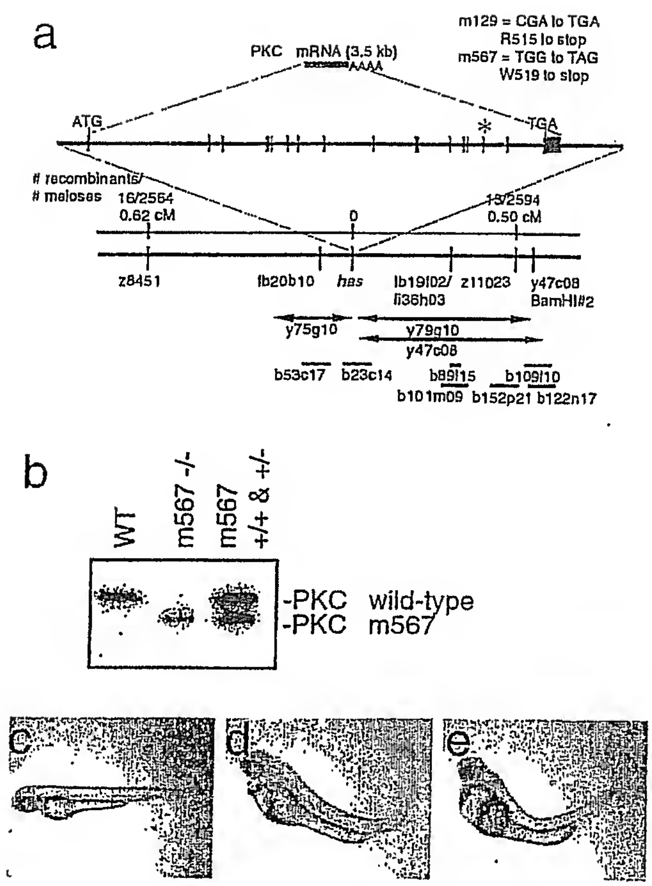

a
m129 = CGA to TGA
R515 to stop
m567 = TGG to TAG
W519 to stop
PKC mRNA (3.5 kb)
AAAA
ATG
TGA
# recombinants/
# meioses
16/2564
0.62 cM
0
13/2594
0.50 cM
z8451
fb20b10
has
fb19f02/
fi36h03
z11023
y47c08
BamHI#2
y75g10
y79g10
y47c08
b53c17
b23c14
b89l15
b109l10
b101m09
b152p21
b122n17
b
WT
m567 -/-
m567
+/+ & +/-
-PKC wild-type
-PKC m567
c
d
e

Fig. 4

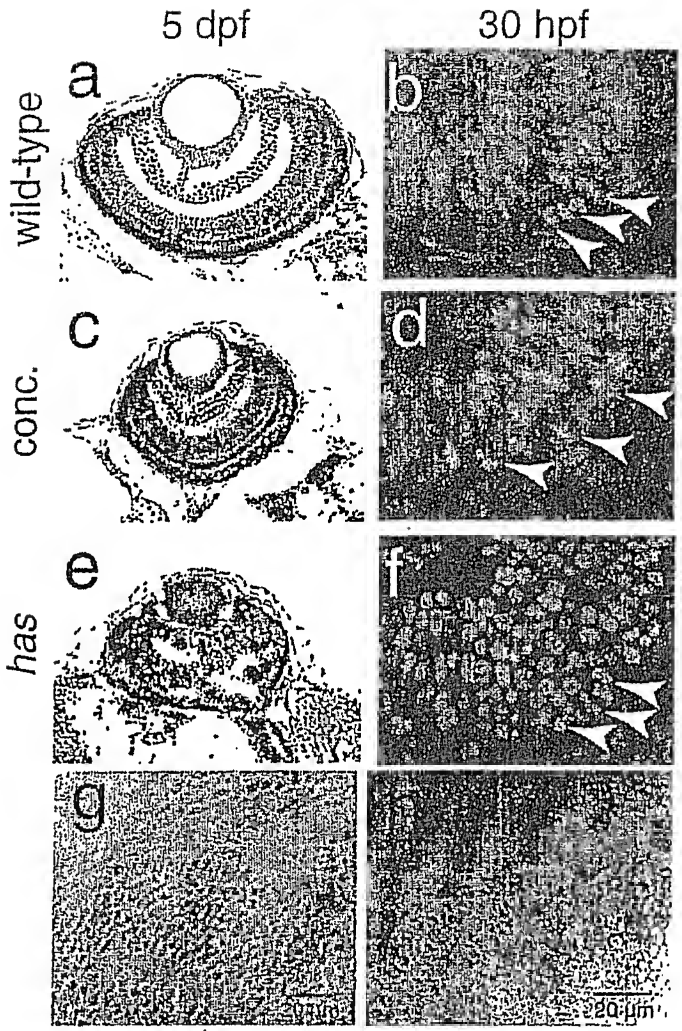


Fig. 5

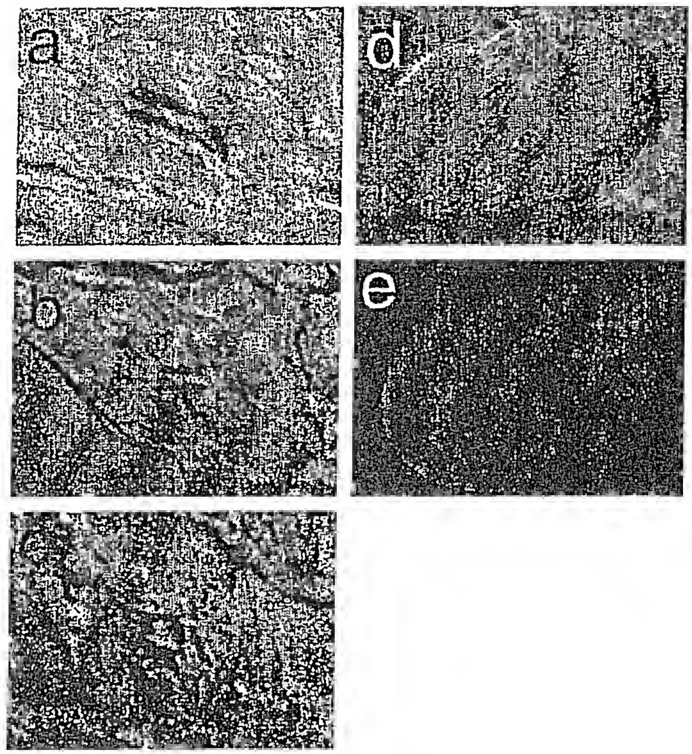


Fig. 6

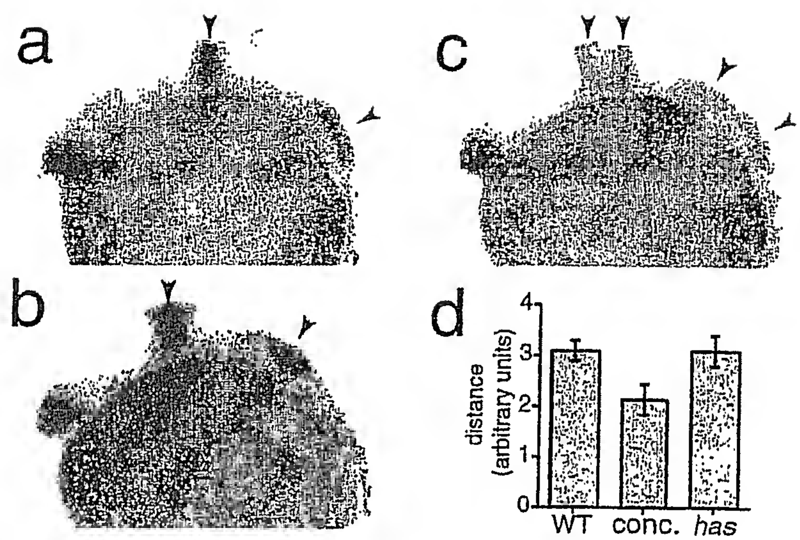


Fig. 7

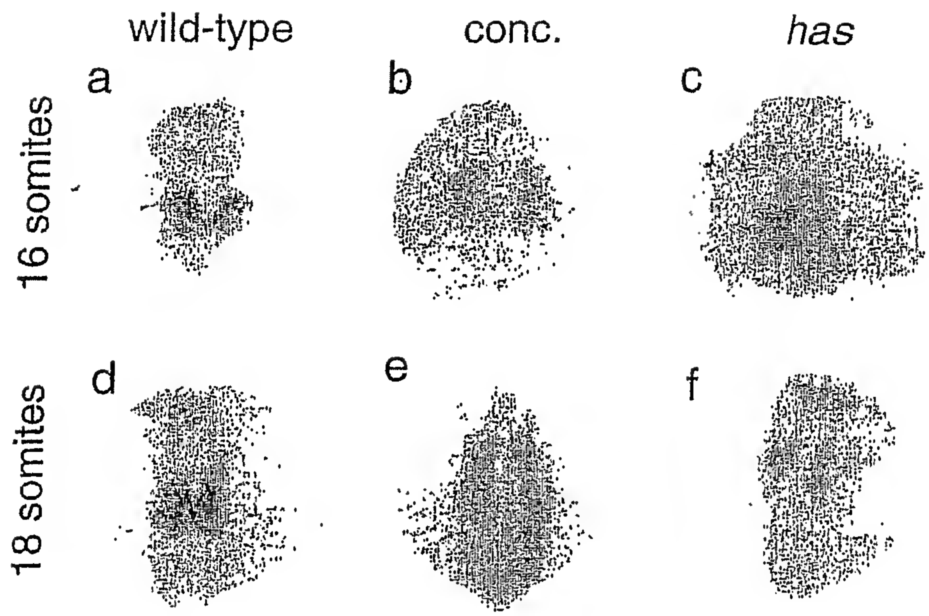
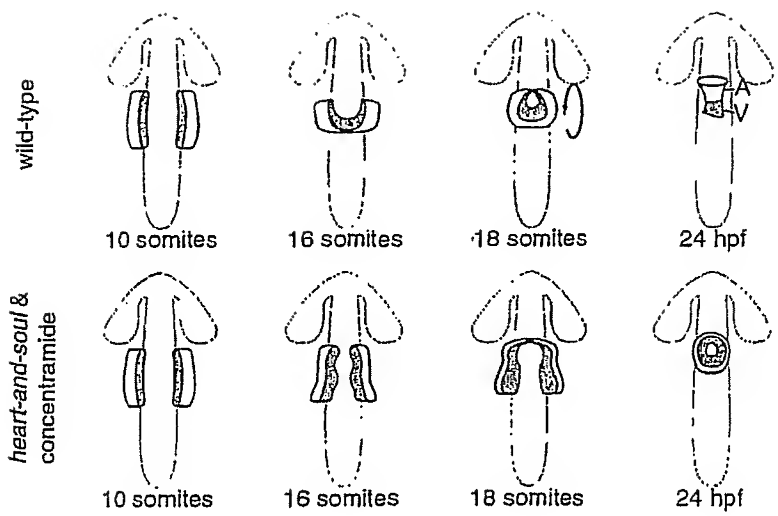


Fig. 8


wild-type
A
V
10 somites
16 somites
18 somites
24 hpf
*heart-and-soul* &
concentramide
10 somites
16 somites
18 somites
24 hpf

SEQUENCE LISTING

<110> The General Hospital Corporation

<120> Methods for Diagnosing and Treating
Diseases and Conditions Associated with Protein Kinase
Clambda

<130> 00786/406WO2

<150> US 60/317,653
<151> 2001-09-06

<160> 5

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 3437
<212> DNA
<213> Danio rerio

<220>
<221> CDS
<222> (143)...(1906)

<400> 1

```
gccaggctgt ttatttaacc ggagacggca ctattgctgt ccaaagaata cgtttagttt 60
taaaactccg gtagtttttc ctcgtcagac gatagctggc tagcatcatt agctaagcta 120
gcaggagtac ggatagtcca ta atg ccc acg ctg cgg gac agc acc atg tcc  172
                         Met Pro Thr Leu Arg Asp Ser Thr Met Ser
                          1               5                  10

cac ccc gga gaa aac ccg cac caa gtc cgg gta aaa gcc tac tac aga   220
His Pro Gly Glu Asn Pro His Gln Val Arg Val Lys Ala Tyr Tyr Arg
                15                  20                  25

ggg gac atc atg atc aca cat ttt gag cct tcg atc tcc tat gag gga   268
Gly Asp Ile Met Ile Thr His Phe Glu Pro Ser Ile Ser Tyr Glu Gly
            30                  35                  40

ctc tgc aat gag gtg cgt gat atg tgc tcc atg gac aat gac cag ctc   316
Leu Cys Asn Glu Val Arg Asp Met Cys Ser Met Asp Asn Asp Gln Leu
        45                  50                  55

ttc acc atg aaa tgg att gat gag gaa ggg gat ccg tgc acc gtt tct   364
Phe Thr Met Lys Trp Ile Asp Glu Glu Gly Asp Pro Cys Thr Val Ser
    60                  65                  70

tct cag ctg gag ctg gag gag gcc ttg cgt cta tat gaa ctc aac aaa   412
Ser Gln Leu Glu Leu Glu Glu Ala Leu Arg Leu Tyr Glu Leu Asn Lys
75                  80                  85                  90

gac tcg gag ctc att att cac gtg ttt cct tgt gtc cct gaa aaa cct   460
Asp Ser Glu Leu Ile Ile His Val Phe Pro Cys Val Pro Glu Lys Pro
                95                 100                 105
```

ggc atg ccc tgt cct gga gaa gac aag tct ata tac cgg cgg gga gct 508
Gly Met Pro Cys Pro Gly Glu Asp Lys Ser Ile Tyr Arg Arg Gly Ala
110 115 120

cga cgt tgg agg aaa ctc tac tat gcc act gga cat gcg ttt cag gcc 556
Arg Arg Trp Arg Lys Leu Tyr Tyr Ala Thr Gly His Ala Phe Gln Ala
125 130 135

aaa cgc ttt aac agg cgt gct cat tgt gcc atc tgc aca gat cgt atc 604
Lys Arg Phe Asn Arg Arg Ala His Cys Ala Ile Cys Thr Asp Arg Ile
140 145 150

tgg ggt ctg ggc agg cag gga tac aag tgt atc aac tgt aag ctt ctg 652
Trp Gly Leu Gly Arg Gln Gly Tyr Lys Cys Ile Asn Cys Lys Leu Leu
155 160 165 170

gtg cat aag aaa tgc cat aag ctg gtc aca gta gaa tgt ggt aga cag 700
Val His Lys Lys Cys His Lys Leu Val Thr Val Glu Cys Gly Arg Gln
175 180 185

gta ata cag gac cca atg atc gga aga atc gat cca ggg tcg act cat 748
Val Ile Gln Asp Pro Met Ile Gly Arg Ile Asp Pro Gly Ser Thr His
190 195 200

cca gag cac cca gat caa gtt ctg ggc aaa aag aac tca aca gaa agc 796
Pro Glu His Pro Asp Gln Val Leu Gly Lys Lys Asn Ser Thr Glu Ser
205 210 215

atc aat cat gag gga gag gag cat gag gct gtg ggc agt cgg gaa tca 844
Ile Asn His Glu Gly Glu Glu His Glu Ala Val Gly Ser Arg Glu Ser
220 225 230

gga aaa gcg gtg tcc agt ttg ggt cta ata gac ttt gac ctg ctg cga 892
Gly Lys Ala Val Ser Ser Leu Gly Leu Ile Asp Phe Asp Leu Leu Arg
235 240 245 250

gtg att ggc agg ggc agc tac gcc aaa gtt ctg ctg gtg cgt ctc aaa 940
Val Ile Gly Arg Gly Ser Tyr Ala Lys Val Leu Leu Val Arg Leu Lys
255 260 265

aag aca gaa cgc atc tat gcc atg aag gtg gtg aag aag gag ctg gtc 988
Lys Thr Glu Arg Ile Tyr Ala Met Lys Val Val Lys Lys Glu Leu Val
270 275 280

aac gat gac gag gat att gac tgg gtt cag act gaa aag cat gtg ttt 1036
Asn Asp Asp Glu Asp Ile Asp Trp Val Gln Thr Glu Lys His Val Phe
285 290 295

gag cag gct tca aac cat ccc ttc ctt gtg gga ctt cac tcc tgc ttc 1084
Glu Gln Ala Ser Asn His Pro Phe Leu Val Gly Leu His Ser Cys Phe
300 305 310

cag acg gag agc aga ctg ttc ttt gta atc gag tat gtg aat gga ggg 1132
Gln Thr Glu Ser Arg Leu Phe Phe Val Ile Glu Tyr Val Asn Gly Gly
315 320 325 330

gat ctc atg ttc cac atg cag cgg cag agg aaa ctt ccg gaa gag cac 1180
Asp Leu Met Phe His Met Gln Arg Gln Arg Lys Leu Pro Glu Glu His

```
                335                 340                 345

gcc agg ttt tac tct gca gag atc agt ctt gcc ttg aac tac ctc cat    1228
Ala Arg Phe Tyr Ser Ala Glu Ile Ser Leu Ala Leu Asn Tyr Leu His
            350                 355                 360

gag cgt ggc att att tac agg gac ctg aaa ctg gac aat gtt ctg ctg    1276
Glu Arg Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp Asn Val Leu Leu
        365                 370                 375

gat tca gag gga cac atc aaa ctc act gat tac ggc atg tgt aag gag    1324
Asp Ser Glu Gly His Ile Lys Leu Thr Asp Tyr Gly Met Cys Lys Glu
    380                 385                 390

gga ctg aga cca gga gat aca acc agc act ttc tgt gga act ccc aat    1372
Gly Leu Arg Pro Gly Asp Thr Thr Ser Thr Phe Cys Gly Thr Pro Asn
395                 400                 405                 410

tac att gca cca gag att ctg aga gga gaa gac tat ggt ttt agt gtg    1420
Tyr Ile Ala Pro Glu Ile Leu Arg Gly Glu Asp Tyr Gly Phe Ser Val
                415                 420                 425

gac tgg tgg gct ctg ggc gtc ctg atg ttt gag atg atg gct gga aga    1468
Asp Trp Trp Ala Leu Gly Val Leu Met Phe Glu Met Met Ala Gly Arg
            430                 435                 440

tct ccc ttc gac ata gtc ggc agc tct gat aac cct gac caa aac aca    1516
Ser Pro Phe Asp Ile Val Gly Ser Ser Asp Asn Pro Asp Gln Asn Thr
        445                 450                 455

gag gat tat ctt ttc caa gtc att ttg gag aag cag atc aga att ccc    1564
Glu Asp Tyr Leu Phe Gln Val Ile Leu Glu Lys Gln Ile Arg Ile Pro
    460                 465                 470

aga tcg tta tcg gtc aaa gcc gca agc gtg ctg aag gga ttc ctc aac    1612
Arg Ser Leu Ser Val Lys Ala Ala Ser Val Leu Lys Gly Phe Leu Asn
475                 480                 485                 490

aag gag tcg aag gaa cgg ctg gga tgt cat cct cag aca ggc ttc gca    1660
Lys Glu Ser Lys Glu Arg Leu Gly Cys His Pro Gln Thr Gly Phe Ala
                495                 500                 505

gac atc atg gcc cat cct ttt ttc cga aat gta gac tgg gat ctt atg    1708
Asp Ile Met Ala His Pro Phe Phe Arg Asn Val Asp Trp Asp Leu Met
            510                 515                 520

gag cag aag caa gta gtt cca ccg ttc aaa cct aac atc tcg ggc gag    1756
Glu Gln Lys Gln Val Val Pro Pro Phe Lys Pro Asn Ile Ser Gly Glu
        525                 530                 535

ttt ggt ctg gat aac ttt gat gcc cag ttc acc aac gag ccc att cag    1804
Phe Gly Leu Asp Asn Phe Asp Ala Gln Phe Thr Asn Glu Pro Ile Gln
    540                 545                 550

ctc acg cct gac gat gat gat gct gta aag aag atc gac cag tct gag    1852
Leu Thr Pro Asp Asp Asp Asp Ala Val Lys Lys Ile Asp Gln Ser Glu
555                 560                 565                 570
```

ttt gaa ggc ttc gag tac atc aac cct ctg ctg atg tct gcg gag gag 1900
Phe Glu Gly Phe Glu Tyr Ile Asn Pro Leu Leu Met Ser Ala Glu Glu
575 580 585

tgt gtg tgaacggtcg ctttatccct ctgttactcg catatcatcg ctgcctttat 1956
Cys Val

ttgcatggtc gcaatcaatc acacgaaagg aagcaacaag aacctgactt tgctttgttg 2016
ggaccagatg aaacagtaac tttgccaaat gtctttcact ttctgccatt tgtaaccact 2076
agtccttaag tgtctatttt tttctcaatt atttttgtat catgttaatc agcagcactg 2136
atgaaaggac atttgtcagt gccttcgacc aacagtttta gctttccgga ctctgcaaac 2196
taaaggaaaa aagaatgact gtgatggtac gcaggacctc ccaatgctaa agatatgcat 2256
tttattttgt aaatatgaaa gagaatcctt tgagcatata tagtaagcca ttttaaaact 2316
ctataccaca tgggatattc ttgaagaaag tttctgatta tctgttttct gtagcgtaag 2376
gatgagaaca ctttgtttta ttactatatt tttatttaag agtactgtca tctatataga 2436
atgtgcacaa tgtgttgaat cagagttttc cagaagttgt tttaagacgg ttggacttgt 2496
ttcctcgttt tagattgaag attgattgga gcagggaaca ttattgaaca ctgttgtaga 2556
tacttacaac tgtgaatgga ggagacattt tctgtataga gaggtgaaaa cacaacagct 2616
ttcttcaatg caggaccaaa ataagacact aaaatgagtg ttcctcttgg cgatctccaa 2676
acagacgagg taaacgcatg ttactactct aactgcagca tgtataaact atttctcgct 2736
ttcttgtttg atttcttgct ctttttcttg tgttaaatgt tatatattgc ctttctggtt 2796
atgatattcc gttgatgtat ttttgcattg aacaaactga gcatcggtga gcattgtttt 2856
tcgatacagt caccgtaaag tggcttcttt cagccctttt ggggatttca agcctgatca 2916
gatgcatgat gaggtttgtg tttactccac acggcgcccg gtttttgggg tgatgcgttt 2976
tttttaaaca tcatgtctgg acgtgttttt tgtttgtgga ctaaactgaa aggacctttg 3036
accataatga ccaaatgatg acattaaaca ggctactcgt atgcagcatc accctctctc 3096
attccactcc atgcacgctt caactcgctt actatttcac agatgttcac accggtttgg 3156
agctgcgagg atctcgttag caacccggcg ttagaaatga ttgaatcgct taaggccctc 3216
gatgcattcc cagaaaaaag aaaatgatgt gctaatatgc tttaaagaag catcgggggg 3276
ctgaattgga cctgtttttt tcttctctct gtatgttttg tgtattaata tgcacactga 3336
aaaacactat caactgactg gaataataaa ctgtaccact tattttgtta acacctcatt 3396
aaagtattta agaaaatctc aaaaaaaaaa aaaaaaaaaa a 3437

<210> 2
<211> 588
<212> PRT
<213> Danio rerio

<400> 2
Met Pro Thr Leu Arg Asp Ser Thr Met Ser His Pro Gly Glu Asn Pro
1 5 10 15
His Gln Val Arg Val Lys Ala Tyr Tyr Arg Gly Asp Ile Met Ile Thr
20 25 30
His Phe Glu Pro Ser Ile Ser Tyr Glu Gly Leu Cys Asn Glu Val Arg
35 40 45
Asp Met Cys Ser Met Asp Asn Asp Gln Leu Phe Thr Met Lys Trp Ile
50 55 60
Asp Glu Glu Gly Asp Pro Cys Thr Val Ser Ser Gln Leu Glu Leu Glu
65 70 75 80
Glu Ala Leu Arg Leu Tyr Glu Leu Asn Lys Asp Ser Glu Leu Ile Ile
85 90 95
His Val Phe Pro Cys Val Pro Glu Lys Pro Gly Met Pro Cys Pro Gly
100 105 110
Glu Asp Lys Ser Ile Tyr Arg Arg Gly Ala Arg Arg Trp Arg Lys Leu
115 120 125
Tyr Tyr Ala Thr Gly His Ala Phe Gln Ala Lys Arg Phe Asn Arg Arg
130 135 140

```
Ala His Cys Ala Ile Cys Thr Asp Arg Ile Trp Gly Leu Gly Arg Gln
145                 150                 155                 160
Gly Tyr Lys Cys Ile Asn Cys Lys Leu Leu Val His Lys Lys Cys His
                165                 170                 175
Lys Leu Val Thr Val Glu Cys Gly Arg Gln Val Ile Gln Asp Pro Met
            180                 185                 190
Ile Gly Arg Ile Asp Pro Gly Ser Thr His Pro Glu His Pro Asp Gln
        195                 200                 205
Val Leu Gly Lys Lys Asn Ser Thr Glu Ser Ile Asn His Glu Gly Glu
    210                 215                 220
Glu His Glu Ala Val Gly Ser Arg Glu Ser Gly Lys Ala Val Ser Ser
225                 230                 235                 240
Leu Gly Leu Ile Asp Phe Asp Leu Leu Arg Val Ile Gly Arg Gly Ser
                245                 250                 255
Tyr Ala Lys Val Leu Leu Val Arg Leu Lys Lys Thr Glu Arg Ile Tyr
            260                 265                 270
Ala Met Lys Val Val Lys Lys Glu Leu Val Asn Asp Asp Glu Asp Ile
        275                 280                 285
Asp Trp Val Gln Thr Glu Lys His Val Phe Glu Gln Ala Ser Asn His
    290                 295                 300
Pro Phe Leu Val Gly Leu His Ser Cys Phe Gln Thr Glu Ser Arg Leu
305                 310                 315                 320
Phe Phe Val Ile Glu Tyr Val Asn Gly Gly Asp Leu Met Phe His Met
                325                 330                 335
Gln Arg Gln Arg Lys Leu Pro Glu Glu His Ala Arg Phe Tyr Ser Ala
            340                 345                 350
Glu Ile Ser Leu Ala Leu Asn Tyr Leu His Glu Arg Gly Ile Ile Tyr
        355                 360                 365
Arg Asp Leu Lys Leu Asp Asn Val Leu Leu Asp Ser Glu Gly His Ile
    370                 375                 380
Lys Leu Thr Asp Tyr Gly Met Cys Lys Glu Gly Leu Arg Pro Gly Asp
385                 390                 395                 400
Thr Thr Ser Thr Phe Cys Gly Thr Pro Asn Tyr Ile Ala Pro Glu Ile
                405                 410                 415
Leu Arg Gly Glu Asp Tyr Gly Phe Ser Val Asp Trp Trp Ala Leu Gly
            420                 425                 430
Val Leu Met Phe Glu Met Met Ala Gly Arg Ser Pro Phe Asp Ile Val
        435                 440                 445
Gly Ser Ser Asp Asn Pro Asp Gln Asn Thr Glu Asp Tyr Leu Phe Gln
    450                 455                 460
Val Ile Leu Glu Lys Gln Ile Arg Ile Pro Arg Ser Leu Ser Val Lys
465                 470                 475                 480
Ala Ala Ser Val Leu Lys Gly Phe Leu Asn Lys Glu Ser Lys Glu Arg
                485                 490                 495
Leu Gly Cys His Pro Gln Thr Gly Phe Ala Asp Ile Met Ala His Pro
            500                 505                 510
Phe Phe Arg Asn Val Asp Trp Asp Leu Met Glu Gln Lys Gln Val Val
        515                 520                 525
Pro Pro Phe Lys Pro Asn Ile Ser Gly Glu Phe Gly Leu Asp Asn Phe
    530                 535                 540
Asp Ala Gln Phe Thr Asn Glu Pro Ile Gln Leu Thr Pro Asp Asp Asp
545                 550                 555                 560
Asp Ala Val Lys Lys Ile Asp Gln Ser Glu Phe Glu Gly Phe Glu Tyr
                565                 570                 575
Ile Asn Pro Leu Leu Met Ser Ala Glu Glu Cys Val
            580                 585
```

<210> 3
<211> 71843
<212> DNA
<213> Danio rerio

<220>
<221> misc_feature
<222> (1)...(71843)
<223> n=a, c, t or g.

<400> 3
aagctttaca ttgaatagag cagaaggaaa gagcatgtcc tcactcggac acctgccagg 60
tttttatttt tatatataca taagtagcct aaatatgcac ttgtcatctg ttaaaattca 120
tccgattaaa catttgtatg tattattagc atttcgacat ttatctgcgt atatacagtg 180
catccggaaa gtactcatag cgcttcactt attccacatt ttttgttaca gatttattcc 240
taaatggatt aaattattgt ctcaacattc tacacacaat agcccataat gacaatgtga 300
tttttttttt aattgttgca aatttattca aaataaaaaa cctgaaaaat cacatgtaca 360
taagtattta cagcctttgc cgtgaagctc taaactgagc tcaggtacat tttgtttcca 420
ctgatcattc ctgagatttt tcagcagctt aattggagat cacttgtggt aaatttagtt 480
gatttgaaaa cgcatacacc tttctatata aggtcctata taagggttga tagtgcttgt 540
caaagcacaa accaagcatg aagacaaagg acctgtcttt ccggatgcac tgtagctgtg 600
agcagatttt tttaaaaaca caaagacatg catttcatct tcaaacttgg aaactctgcc 660
tactgagaaa tctcccaggt caaaaatcct ggatcatata ctgatgataa ttatcgggtc 720
taactcttct tcagactgtg tttctctctc tctttccaac acacagttgc tgaattcatc 780
cactcgagcc agatcaataa ttctcctcaa tcactctcac tctctcccat aaacacacac 840
tcagtggcct ccgctcccct tatgtaaaca gagactgatt aggtcattct gctgcttatt 900
ttgagggctt gcgttgcatc tactggagtc tctgatattt acactggagc agacctggag 960
ctcaacaccc tccatcaaac agacaaaacc gcgaggctct tccacctctg acctttcatt 1020
acccttcaat atcttaccaa acacatgctg gatgaagata aagctccaaa gacacaatgt 1080
catgctaagc aaattcctga aaatgccatg tttgcagagg tcagacaggg agtataaaat 1140
aaagcagaaa tctttgtaaa actggactga aggcacagtc atattaatcc ttttctgcta 1200
aaagaaaaaa aaattaggaa gttaaattca gtaatcttaa caggaaatca tgcaatcagg 1260
tgttatttgg ttggaaagtg acttcagcgt gtgcgatgct ttatttactg ctttttgaat 1320
ataatattta gtatgtgtat tttgttaata actgaacaag tacacaaata catgatggta 1380
tgtgtgtggt atatggtatt aggcagtggt tagaaatcaa ctgtaagaaa catctgatca 1440
taaagatatg attatatttg ttgaagcttt acaattcaac gctacttcaa gttacatctt 1500
gtcttgctta aatttaccat taaaaggtcc aatgagatta agcgggcttt tacacttggt 1560
tcaattgcct ggaccgaacc taagttccat ttgcccccct ttgccacctt ctctgctggt 1620
ttgtgttcac acagtctttt ttccttctga atccaggtac acttgcatca aagagcatag 1680
aataactaag cggcgacact agtgtgattt gggaaactcg ccgcggcatc tagtgtctca 1740
gcggccattt tggaatgaaa attccaatag aacaagtctt tagcatatta taagtctgta 1800
aaataaacta ttaaaagtgt tgatgattgt gttagtaagt gttatattgc catctttcag 1860
gttcaatgtt aatgcgcttt ttaaataaat aattaaaaaa caaagcagct gcttgccatc 1920
gcgacagcaa aaagaacaaa tcgatggacg attacttttt ctccaaaatg gcagaatagt 1980
aggaggatat ctaagttagt gtgcccaaaa cagtgacaaa atttgcattt agaagatata 2040
aagctgatat aaacatgtaa agcttgtaat ttgtcacttt cacatatata gagcaaattt 2100
cctttgttgt tttacaaagg ggaggagcta atccatgtcc cactctctct tcgtgttttg 2160
gttgaaataa cgtcaaacat caaataacaa tgcacgtttc gaaacaattc aagtcagtgt 2220
cagtttcact tttaattaca attatcatca ccatatatcc agtcaaactg gaggaccaga 2280
actagttcaa gggtggtagg actgggcgat taatcgttaa gtagtcgaaa tcaacattta 2340
aaaccactaa ttgttttaca catttttgaa tgccttgaga cagcatattt tccattacaa 2400
taaaatgatc atttattcaa aaagattcaa aacttaaata ttatttacag gatacagata 2460
ggttatttta tttagaagag atgctgctta ttttatactt tgaatatgaa aaacgttgaa 2520
aataattcat tttgtttcca aaagagcaag ttatttattt atctttattt tttataagtt 2580
ttaggcaatg tgtgttttaa tttcagttgt tcaacgttga agttcaataa ataatcagag 2640
attgtagctt gtgcttcgtt cacttatttt gaaatcaagt aatgcgccct tcattcaaaa 2700
atctcttgtg atatgtgagc ttatttaccc tataaaactt aaggaactat ggaggacaaa 2760
aaataaaata aataaacgaa ataatcgttc atcaatcgta atagagttaa aatgttcaat 2820

taatcaacat ttgaggccaa atcgcaccag ccctaacaag tggtgtgtct cacatgacta 2880
aatggaaaca gcaaagcaaa gcaaagcaaa aaaaaaaaag gaaatgcagc tattagatag 2940
gaagcaaaaa atcaaggggg gtaaaaaatc tgacaacgaa ggtatgtcag agatgaggtt 3000
tgcggttcga gctacaggat ggcagaacaa aatacattag gcaggaaaat gacaggtttt 3060
ctttcacaga tttaaaatgg aaggaaaaaa aactgaaagt ggaaataaga gcacgatagt 3120
ggaagaatta gcctcctcgc ctgaggtgca tgaagtcact gatttcacaa ttacaaaagg 3180
aagacagaga gacgcaaacc gagaaagaga gagcgaaaga ggaagagaga gagaggcagc 3240
tgtctggtgt ctgcaccgca gtcgctgtct catcctctgt gggagatcga caggactcca 3300
tctgtctgcc agacacgtct acacggcaca ccagccctca aaccattaca gctgcatgtc 3360
tatcactgtc gtacaatcaa acaaacacac atgagctaac tgtatgccac ctaagtggga 3420
agactaggac agctaaatta atgggatcat gaaaactcca agtacaggca catgcccaaa 3480
tgactgcgtt cacaccaaag gcgtcgagag catccaagta gctggaaatc attctttttc 3540
aatgggagcc tgcagcgata agcgacaagt cttcacggtt gtgggcgtcg aggagagttg 3600
aaataaagtc aacgttatag taatgtgctg tgatgcagtt cagtggcaac caatcggaac 3660
aagtcatgtt caagtccacc gcttgagagg agtccagaga acacagtcct gtgaactttg 3720
gttctgacca cagttgttcc caagggtttg attattgcgg ttactggatt tccaattatt 3780
tacaaagttt tcttttagga aatgcgtgat gcgcattaat tttttggttt tttccccttta 3840
aaaaaaaagc aggtatctca tgctaactac aacaacttaa tattttcgac atatggacac 3900
atattggata agtggagcaa aaccacacca catgaaacaa cttcattttt agttgttaaa 3960
ttaatttgat aaaagggaca caacactttc acgctagaaa gcatgttctt ttaatgtggg 4020
attgtaactt taaatacaag attaatgtag tgaattctcg cggccggagc tgtaaagtca 4080
gacagcgttg tcgccagggt ggccagagtg aactttgaca cactcgccac cctcggtgtg 4140
aatgcacagt gacttgcatc gtttttgaca agtgccaagc agactactga aatcatttta 4200
gaaagaaatt ctaaaatcaa tctaaatata aactggggtg agaaagctaa atatatttgt 4260
gtttggtaaa taggaaaaaa aagcagtgcg acaagtgttc caatagtgtt tcttgtcagg 4320
tggtccctat atagcatatg agccttaacc actcgtgctc tattgacctt attgtaaaat 4380
gcggaagtgc gcctgttttg acgattgttt tagaaattac gattcagtcg cctatgggag 4440
aaatgactag gaataataaa cggcagaaaa cgatcaaact acttgctcta caaacaaatg 4500
tttgcatgac tatacagacc aagtagaata atataataag aaaatatcag gtttgcaaca 4560
tcaaacagcg aaacgagcag tttttaatgt ctaaaaataa atggaagtga atgagactgg 4620
aagtctcaag ccaaaaagat tcaaatggct gcgccactcg tcggccaaga ataaggtgaa 4680
tagacaccct aaccgaatac agcgcttgac aacacaaata aatacagact tacaaaaaca 4740
caaaaactgt ccaagaaggt tcctgcgctg aattctctgg agcagcagct gtgcatcaaa 4800
acctagccag gcagcatctt taagcatgca catttcacca aaatgaatac aaaaataact 4860
ggacatctct taagttctaa aactaaatat tttttacttc attattattt acagcaaaat 4920
cctctaagta aaattcactt agtttagata gtatttgctt cttctctaaa ttaaattaaa 4980
gttatacaag ttaatgagac aacgaaagga ttaataaggt gatgattgtg cactgatgat 5040
gaacacctgc tgttaataaa caaacacaaa gagaaacaca aaactacaac tgacttcagt 5100
cacagccttt gataaaatca actgaaatat aaaagattga atctctcaaa atctcagcag 5160
aggatcatta agcaactcaa caaagagcaa ctttacttat aattgtcctt tgagggacca 5220
aatgctctct gaactgagta caatatactc agaggaattt gctgtgtaat tgtaattaat 5280
atgttattat tgttgctatt tgagggatat ttaaatcacc aattttctca gaaatatgct 5340
ccattttgta gttttactaa gctttttgat aatgctctga aaacaaatat atatatatat 5400
atatacttat ataaataata agcaataaaa tcttaaaata tttcaggcat gtttcaaaat 5460
ctagcgatct gtcaacctag gttgaattta agggccttat agactatgcc accagaatat 5520
gccacctcct ctggtcaact cactagtttt aaaaaaccca gccagtgttt ccaaaagcac 5580
agcagacagg ggagtcagtg tccttgataa agtggaagag gactgcatcc gagcagactg 5640
aagtgccgtg tgctgaagtc ttgttatgag ggtctgatcg tcaccaacat ccacgcggac 5700
acctgtggaa actctgagtc acttgttctg ccgctccggc atcacagatg tttagagtta 5760
acatttcacc cagtaaactg atgagcctgt ctgccctgct tcccgtacac acacacacac 5820
acacacacac agacgcccgc ataacagtga cttcttgatt tggacaccag cagaagagta 5880
tgcatatgtt gtttctcttt agcggctaga cccgtgtaag ctcagcgtct aggtaatgac 5940
ctttcacgta agcacatatg cctgtttacg gttcctccaa aacagtttaa caaatcttgc 6000
aagcgcctgt gtacttgaca ccacttcaat cttaagcagt gattgttaat ttaaaagtga 6060
ggaaaaaagc gagatacaat ttatgtttta aaaacttttc tgccttggca ataaactgga 6120
acaagtaagt tgaagacaag tttagcagtt gcaaatttta gttggaagtt attaatagaa 6180
tatttttgaa cattgtttta gtaatctgtg aattaatagt aattgtgaag ttaagaaatg 6240
ctttattttt attttagtta gtatatttac tttaaaagca aagcaaagat gggaaatatt 6300

agcttaagtg tttttgcaat cttattttta aatattttaa tttaatctta cttaatttgt 6360
aaacttatca gatacctaga atttaataaa attttattgt tgtatcacaa ataggcatga 6420
gacgataacc attttcaagg tataacacgt ttggaatttt tttttagga caacagtatc 6480
tccagcagaa aatatatcca agatgccgtt ttaaatggta aagggaagaa acaaagtttt 6540
agaaacaaat taaaacagaa gtcaaggaat catttgaatt atttaccctg acatgtttac 6600
tgctctaaaa tattttaaat gtctcttaaa atataatata atataatgta ttcaaaggag 6660
aaaaaaaaac tttcagtttt tacccagaca tttaaaaaga acgtatttta gagcagtaat 6720
cacaatacag tcaaagcgtg atgtttttat ctaaggttat cataccgtta gaatcttata 6780
ccggcccatg cctaattaca aggaaaacat agcgtgccga taaattgtaa aaatcataaa 6840
taatttttaa aacaacatat atttgtgtat gtaagatcat aacgttaaaa aactgtgtta 6900
gaacatgtaa aacacaataa aaggtattgt ttatatgaaa atgaaacctt tttttttttt 6960
ttttccactg aacgcaaaaa aatacatttt caagaaattt tcaagaaaac cttaaaccat 7020
tcacttctat agcaggaaaa acaaatacta tagaagtcaa tggttacaag gttttcagat 7080
atctacaaaa aaaaaagttc cactgttttt aatatacagt gctcagcata tataactaga 7140
cccctcacaa atctatcttt caaatttata attttaatat aaagctatac aatattatat 7200
ttatgcataa tacattagat tagtcagtac tgaagccaaa tttggaaatt atctaacaaa 7260
ataacttacg ataatgatac aaaaacttgt acacccaaac acatatgtta tagagaaaca 7320
ttaaatacaa attttaaaat gaggaaaaat caagagaaga aaaaaaattg aaaaaagttg 7380
ttgatatatt gtagtttgta catttttttt ttttgcattt ttttttgctt gaatttaact 7440
gtattatctt tcaattacta aattggtttg gtgactaaaa tactatttta ataaacattt 7500
ctgtttaata aatctgtttt gtttaaatgc accaaaatac attgcctata ttcaccgaga 7560
tgtgaataaa attattgatt gtcaaaatgg ggtctactca tttatgctga gcagtgtaaa 7620
aaataaacta aagacaggtg tgaaataagt gaagggtgag taaataagga cagaatacat 7680
ttttgggtga actatccctt tttgggtcat tgcatcttaa aataaaactc aaagttaaga 7740
gcttacagga agtaaaaaaa aaaaaggaaa aaaaaagcat gaagtcaggt gcaagacact 7800
gccagacagg gacgtgtcgg gagttcggca gttaacagct cgctgtgcaa agagcaggtg 7860
tcagtgactc actcgtgttt ctgggagttt gatatctcac cctcagctga gcggagcaga 7920
gacgctctca ggggaagttt agcgtgtgcg tgtgaatcaa tttcattagt gtgcgttaag 7980
cagaagtgag ccgagagtta cagtgtagct ggctgtttgt gtgtcagggt gaggtcagtc 8040
tgtgtagcag atgtgactgt atgactgaca gactcaaggt ctgttttagt acatgaagtg 8100
tgtgttgggg gagggtggaa ggaggaatgc ggtttgggtg ctagacgaga caccagacgg 8160
tatttccggc acacttgcat aagcacacag gagagactga ggcttttctt ccgcacacac 8220
agacagaaca caatcatttg ctctcgtcgg aacggaagaa aaataattgt gtgtgctgga 8280
acattacagc acagttaaac cattaactgc tgccacttgc attcgaaaaa gaaaaaaggg 8340
actctggcag caccgacagg gggtcaatct ttgtctaaca tgagcacaaa aaactagaaa 8400
agctctggct tggtcaagtg ggtcatgaga catggctggt caatgggagg gaaacaatcc 8460
agatcttttt ttgcactagg ttgacctttc tatagattct acagcacgag gacactttgt 8520
tgagttttgt ttcaaaggtt gcattatgaa agtggaagaa tctccagtgt tctgcttttg 8580
gattttgcaa ttacacaatg caggaatttc cccatctaaa gggacaaatt cacagatggg 8640
aaaatataga actttatttg tcaacactga tttcctgtca gtgtgcaaga ttttcgaaac 8700
attgtagccg ttgcagatta ggcatgggac aatgacattt tgaaatgaat tttttttttt 8760
ttttactttt ttgggaaaac agtatctcca gcagaaaaga tattcaacga tgctgtttta 8820
aatttaaaat gtaaataaat tttaagagta atgaaaacag caaaagtcaa tggttcattt 8880
gaactattta aggctgattt atacttctgc gtcaaatgca cgcgtatgct acggtgctga 8940
cgcatagccc ttcaccgtgg ccgttggcga cgctgacgtg cacctctcaa aaaatttaac 9000
tacacgtggc aatgacgctt agcgcaagct ctgtgattga tcggcttggt agcgctgacg 9060
agtctgggtg ggaccgagag ccgcgcaaat ggtgcgagcc tgatagagcg attgtttaca 9120
agtgtggagt tccgtgaagg agctccggat ggaaagtttt gttttgtgtt tacctcatag 9180
ttaaagttgt tgtacgtccg ccagttcctg cctctaaatg agcgagtttg agccacttgt 9240
acatccccga agcgttcagg aaaagcaaaa cagtgaagaa actcgacaaa gaggaacatt 9300
tacacctcac tgccaactag cgtttcggaa gtgttaatgc agatcaacag agacagggcg 9360
cagacatata aatacacagc tacgcgcgtt acatgcgccg tgggttacgc aggtcacttg 9420
acgcagaagt ttaaaccagg ctttagccta acatgtttat tgctccaaaa tattttaaat 9480
gtttctgaaa acaaaatata ttggctaaaa tcgcctacta ctcagtaggt actgcatctg 9540
aatttaaatt tacaacccga caaaaacgga cgttctatac agtagtaatg tggctagtat 9600
gaatggagct tggacgtagt acagatgcca ttttgtcatt atcacgtgac atacccgctt 9660
cactcccatt cataaattct ctaacggtgc atcatgggat agcgtagcgt ccatcggata 9720
cacacttcag aatctcaccg cactctaggt catccggata cttctcacat actgatttc 9780

gaattctatg aattcaaaca tactactcgg ctcgcatact gtttttagca tatggaagta 9840
tgtgattttg tacggatcca ttgtgttcaa agcgaaaaaa aaacgttgtt gctttactca 9900
gacatttcaa aagatttttt tagagcagta atcacaatac tgtgaaacca aaccatgata 9960
tttttatcaa aggttaccat accgtctgaa ttttataccc acccatccct attgtagata 10020
aaatactaga agtaacaaag tggtatgtgt gcagaaaaac actgttcgtg atcatttagt 10080
acaatctcag actggaaatc agaaaaaagt tatttaaatc catttccttt aaacttaaca 10140
ttgccactag atagactaga ttaaatacaa aaatatatat tattatacat tatattctta 10200
tcatctcatt atgcatatat taattaagga ttttggatgg aaagggtaag tgtaaaactg 10260
ctgttgcata tttttttaatg ctttgaatat ttaaatacag atttttgaat tgcaatttat 10320
aagaaaaata aatacaggta ctagaaaacc ttgaacatcc aaggtaacaa aattccacca 10380
ttttaatgtg tgctgtcatc attcaccccc taaagctgac cctgtactga cctgagagga 10440
agataatgta cttccccttc agccagacca actggaattc cctccctccg gccatacgtc 10500
ccaaccaagc aagcgtcacc tgctcgtgcc accccataaa attgggtcag cactagttta 10560
tcaaaacaac tgcagccttg cccagacaat agcaaacagt ctcctctgcg gactatcaca 10620
cattaccatc tggtcacaca cgtgcaaatc cacttgtgaa catgaagcaa aacttgacct 10680
tagtgaacca gaacacatgc aagacaaaaa gacgcttact ttgtccagag gcctctttag 10740
ctggtagtga aacttctcct tcatctcgtc cagcagctgc ttgagctgtg gctcctcctg 10800
tgtgccctca tgcttgcgcc ccagctgaag gtagcagggc cacttggctg aatcgaagcc 10860
ccagtggcag gtcctcttgt caggggactt gtgtgagtgc attacaaagt tctgtgggga 10920
aaacagcagc tggcactcca tgcactgaat gcaaggggca tccggctgga cgtagaaatg 10980
gggcacaaac aggccttggc acttgccaag acactggtgc tccacctgga aactggcgtc 11040
actctccttc agcaggcctt ggcctggcag tttgctgttg ggatcggctg agatggtggc 11100
acctgggcga agcagggcat tgcagagccg ttgggcatca gttagggtaa tcaggccaca 11160
ggaaggtgca ttaaaaggca aaatgcccag caccttgaga atgtgcagct gctccgcatc 11220
acaccgcgaa cagtaaacgt aaagttcatc acaaacagca ttgatctgct gcagagagaa 11280
gtctcggagc actgaattga gtacctgggg cagacaaaga cgcttctcac cacctacaac 11340
aaagcaggag atggactctc cttcaaggag agaatgggta agctctgtgg agctgtcaca 11400
gggcactaag agtgggcctc ctccgaggac tgggggagac ggtaggggag ccatgccagc 11460
aggagaggcg cactcctgtg ccgattttgc tgagaaagca gcaggacctc ccagggagct 11520
ctggctgctc agcgtgaact gtgccagcgt gtgcttgagg ccggcgctca gatctaaagc 11580
cttggatgct ccgacaatgt ggaattctct ttcctccatg tccatttctg agctggtgtg 11640
ctcctcacgc tccttcttga cctttagggg tttggggatg ctctccaggc tgcgacgttt 11700
caaatgcatc tctgccatta cccgtttttt aatgggagca tcttccagtc gctccctgta 11760
cagcctcttc atgttgcctt taaatgaggt caggtcttta aaaggggttt tcagacctgt 11820
ctgagggctg gccatatcca ttcacagata actggtcttc cttgggtgga aaataatcgt 11880
cagacacgac acctttgggt tggtcctact ttgctcgctg tgttaaaggt tgcaaatcct 11940
tttacgtcta gctacatgca tgatgctgac agaattaaat gttcaacgag cttcctgtga 12000
atgaaaaggt aaaaaggctt gtattaaaat ataattctta ctaaatcaaa cacaaatcag 12060
aatgtgaagc cacgtgaatg cggatgaatg gaaaatgctg ccagagcaat taaaaggcat 12120
ccagaagcgt tttagcgttg ggcacagtgc taaactagac gacagaaaca ttttcacctc 12180
atcaggataa tctaatttga aagcaatcga tctaaggcca gtttctcgct ggtttagtgc 12240
gctgtttttc tctactgaca acatgccgaa tccgtcaagg cggtaaatga gtcagtgcac 12300
taataacgtc ggtttaacac acagataacc aacagcatca gtaactaaca gcgagcagcc 12360
atttagtcct gtccaaccac agcaggcttg aaacaaaatg tattaacgac acataaacac 12420
cacttctgcc acgcaagatt aaatgtattt attatattgt aaataaaaaa ttatgttttc 12480
atcgtacaag caaaagcctg aactggcgga atttccgagc acgaatatga aggcaaaatg 12540
aaagctcagc ctcaaattca gcgcatgcca gtggggtatc ggcaaaatcc atcagaaaaa 12600
cgcgtttaat ttcagaatca caacatgcgt ttctgttcta caaacggaat gcattcattt 12660
tggcgaaaat taaagggcca ccaaagcata ataatctcaa gttcctcgaa ataaacgctt 12720
ggttttgctt taacgttacc agatttgcgg gtcaactcaa acagactgac cctgaccgca 12780
gacgtcgttt catttcctca taaaataacc agtttccaga aacactggca aaacgcggtc 12840
tattttttaac cctgaatgac agcgtctctc tttttacccc cgcaaggaca ccgcggctct 12900
gtccccgcct ctacccgtct ctcccctctc cttctccggg cagagctgcg ctgagcaccc 12960
gcggtgtgac aatcaccatc cccgcaatcg ggaaaaccct tcacatgaaa aaaaaaacct 13020
gatcacggct attacacctt aaatatccca acacgacatc taaagcttct attttaaatc 13080
cattcccatg tcttcattcg gcagtgaaaa cgtttgtcaa ccattcagcc agtctgacac 13140
ctgaccaccc cctcaaaata cgttaaacag cgatatcccc aaaactgttt ttgaaaaaca 13200
gaaatacaga catttgtatt aaacacaata tagatcatat ctaagattta aatcaatcac 13260

acaaatactc ccagctcgat caccaatatg accaggttaa acacataata cacacgtata 13320
ttatgccctt ttaaatggca catcaaaagc accaaaagca ccaaaaccgg tttaacatct 13380
gaaaaactgg ctcgttcgca cagacagctt tagcagtgcg gctagaatat ttagaccctt 13440
tagcacgcgc taaccgagct aacgctacaa atccccgttc agagacagaa ataaacgtca 13500
cttaactatt acaaaccttc aaaacggttt taaaaaacac agcgacaaac tgctgaacca 13560
ccagagtaat ggacgcagtg tataggcgaa tcagatgtgg gttttaaagg gacttgcctc 13620
aggatgaacg atctgctctg ctgtctctct cggtctctcc tctagtctgt ctgagcgtca 13680
acaacacgcg cacacactca aagccttcag acagatcgcg cgtcacacac agcgagagcg 13740
cgcactggcg gagcggatga tgttgcgcgg gcgtgtctat gtatgtgtgt ctgtctgtct 13800
gtctgtcctg tctgtctgca gcgcgccgcc agattctcat gaactttgca agaatttaca 13860
ggttacagta aatgtaatgt aatgcatgta atacatgttc agttgtcatc tgtggtctac 13920
tagtaacttg cgcttgcaca catgactaac caagggacat ttctttagaa ataagggagt 13980
tttgctgtct acttagggac agtcatagtg gagcatgctg aatttgaatc actttatggt 14040
aagagaaagg agtaaatgga aattaaatag taactttagc cacatcccca ttcacgctat 14100
gacttagcat gatttttgag gaataatgaa tggcagcatg agctgaggtc tgtcaatgtt 14160
tcacagataa tgactcatct gaaatgggct gtttaaatga aatacatatc atgcaaaaca 14220
tcacagtttt tgtacttggt aaagacataa aacattattc tgttttacag aattatagtg 14280
ttgagggcgt aacacattac aagtaacgag ttacgtaata atattacttt tttaagtaat 14340
gagtaatgca tatttttaaa aattaagtaa tactatttga gtgacttttt agcttaatta 14400
attagcttat aaaaacaaat ggctgaaata aaatcaatgt cagattgaat cccacactca 14460
atgatagaat gcaggaaaag acagaaacca acatggcaga gccttatatt tctgcaatgg 14520
gagttttctt attattctga acgaatcaca gaaaaggaaa acaggattat agtcttaatg 14580
gatagtgatt ttactttatt aatagcacaa aaaagacaaa agtagtagtc acattgcatt 14640
agactttcat tattacctgt ataggcatat atccttgagg gtcaggaagt tctcaaaaga 14700
taagattatc ctacattatt attttatgat gtgtttttaa aaggtaaatg aaggtgtagg 14760
ttatttagtg cgatgtttat tgcagagctg ctttatttta aaaaagaagg aaaaaatcct 14820
gcaagatctg aaacagatca agtctcagcc aggtcagaaa aggtaatgca aaagtaactc 14880
aaaagtagcg tgacacatta cttattataa aaagtaactt agtaatgtaa cttacttttt 14940
tgaggagtaa ctaaaaattg taatgcatta ctttgaaaaa taactttccc caataacaat 15000
ttacagttga agtcagtatt ctagccctcc tttgaacttt gttttctttt taaaatattt 15060
tacaaattat gtttaacaga tgtctggtaa tatttttttt tgttctggag aaagtcttac 15120
ctgttatatt taggctatta attcaggagg gcaaataatt cttacttcaa ctgtagttca 15180
aaagttttag gaaatgggtt tcagaaaatc atgctttttt tcagtaaata ttcagctttg 15240
aatcactggc ataaatagca ttttaaacct attcttaatt aaaatgattt tcatttatat 15300
tatttctaaa ccaaaaaaaa atgtataaaa tatgacaaaa attgcgaaaa atgacttaca 15360
gattttattg tatttattta caaaaaaata cccatttagc tttgtttatt ttatttctat 15420
ttcagtttta gctttaattt ctgtttactt tcaatgtgtt ttgttaagtt gaaaaattat 15480
tagttttttt tttttacatt tttaaaatat gttttctat atttccattt tacttgtaat 15540
ttttataata cattgtgtat tgtgctgtca ttgtttctta aactatttat tcattcattc 15600
attttctttt tggtttagtc cctttattaa tctggggttg ccagagcgga atgaatcgcc 15660
aatttatcca gcacatgttt taagcagcgg atggccttcc agctgtaacc catctctggg 15720
aaacatgcat acagtcattc acactcatac actaggccta tttagcctgc ccaattcaca 15780
tgtaccgcat gtctttggac tgtgggggaa accctaacgc agggagaaca tgcaaactcc 15840
acacagaaac gccaactgac tcagccgagg ctcaaaccag caaccttctt gctgtcaggt 15900
gacagtgcta cctactgcgc cactgtgtcg ccttaaacta tttttattg ttttattgtt 15960
atttttggat ttgaaaattt gtgaaaatta aatcacatta aacaactaaa ctgaacttca 16020
tctcagaaaa ctgggctgtc attttcaatt tactagaact tctatgttaa gcaactttaa 16080
tagaatctac attgtaaaag cgctataaaa ataaagattg tagttttaat aatttatcgt 16140
gtactgtact gtgctgtcat tgtttttgta tactatgtta gtttgaattt ttttatttca 16200
aatttggttt taattcagtt ttagttttac acctccattt gggaaattaa ttccaagttt 16260
taattatttc acctataagg atacattttc agactcatta agttttgata aaatatttta 16320
tttcattttg cattttattt cttgttcaca tttttaatta ttgttgtcct tttttatttt 16380
aatcgaaagt tgtagtaagt acaaattccc catgtccact tcattcattc atttatttat 16440
tttccttggg cttagtccct ttattcatca ggggtcgcca cagtggaatg aaccgctaac 16500
ttatcaagca tatgtttcac gcatgaactt ccagctgcaa cccagttctg ggaaacaccc 16560
atacactctc acatacacta aagccaattt agcttattca attcactttt agcgcatgtc 16620
cttggactgt gagggaaaac agagcacttg gaggaaaccc gtgcgaacac agggagaact 16680
tgcaaactcc acacagaaat gccaactgac ccagccagga ctcaaattag tgacctactt 16740

gcggtgaggc gatggtgtaa tcaactccac tcaaatgaaa tatattgtga agtaaagtct 16800
acgcatcaat ccacagaaga tttttcatct cactctgtgg ccataacagc ttaaaaagaa 16860
ctttatcgga tttgatgtcc gaattaagct cattttaaac cacccagaca tatttatgga 16920
cattagtgtg tgagtaactg ttacgctagt acaaatgttg tggaggcttc agccaaggac 16980
attaagtaat aacactagaa cattctctat ggctgagtaa tggcctggag ccaatccagc 17040
atttcgctcg gtccacaggg cttcaaccat ccaatggcct tcatgttttt gtcaatggac 17100
ccatgatggg gtgctgggat cacctttgct ttggtttcca tctctgtgtg agccatttgt 17160
taggtgtcat ttaaatgtta aatgggagag gcagagcatt cagcagctcc ttcacaagcc 17220
cagagcagca cattaacact tttaaaagct cctcctccac actgacccag gtcatgttca 17280
ggcaagacag ctttcgggag attccaacag ttttgatgtt tgtgctccta ggtgttgtgt 17340
tttttcttta gacttaaagc tcacacaaaa gagcaagggc ttatgttgca aaatcttaca 17400
tttcacttaa aaattgtctt gttgttgcgg aacggcaaac ctttattgga ataatagagg 17460
agaaaataca tagtgtgtgt gtttaataaa gtgctttcaa ttaattaaag aaaaaaacta 17520
atttagagat ttgtataatt aggtttaaat attaaaatcc aaaaattttt aaattatatg 17580
taaataatta caaataagca aaaaaatgct caaaatagag tgtatgggtt aataggttgt 17640
ttaaatgaat aatatacatg cacgtatata catttttatt agtgtacttg tatactataa 17700
caacaaaata ttacactacc tgacaaaagt cttgtcttct atcccagttg taagaacaac 17760
aaataattac ttgtcttgta gttgatcatt tggaaaagtg gtagaaggta gatgcagaaa 17820
gtagattttt cagatgaatc atctgttgaa ctgcatcacg atcatcacaa atactgcaga 17880
agacctattg gaacccgcat ggacccaaga ttcttacaga aatcagtcaa gtttggtgag 17940
agaaaaatca tggtttggag ttaaattcag tttggaggtg tggaaaagat ctgcagagtg 18000
gatggcaaca ttaacagcct gaggtatcta ccctgaggta gcctgaggta ttacaaacca 18060
caagagaggg taaattcttc agcaggatag aactcctcat acttcagctt ccacatcaaa 18120
gttcctgaaa gcaaagaagg ttaaggtgct ccaggattgg ccagcacagt caccagacat 18180
gaacatattg agcatggggt aagatgaaag aaaaggcact gaagatgaat ccgaagaatc 18240
ttgatgaact ccgggagtcc tgcatgaacg ctttcttggc cattccaaat gactttatta 18300
ataagtgatt tgagtcactg cagagatgga tgcagtcctc caagctcatg ggagtcatat 18360
acaatattca ttcttttacc accgcaccat gacattatat tctatcctga acattatttc 18420
tgttcagacc ttactgtctt aattaaatca ttaaaaatca aagcatgatc atattttatt 18480
ttggtcaaat aagcagaaat tttaacacct ttgactttca tataaatcac ttctgtaacc 18540
aaatgatcaa ctagtagtaa agttattatt tgttgttcct aaaacttgga taggcgacaa 18600
tacatttgtc aggtagtgta aatatatata tatatatata tatatatata tatatatata 18660
tatatatata tatatatata tatatatata tatatatata tatatatata tatatatata 18720
tatatatata tatatatata tatatatata tatnnatgta tntannnnnn nnnnnnnnnn 18780
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 18840
gtctaatctc cttggtgtta atatctatcc atccacccat ccatccatct catccatccg 18900
tcacttgctc aaaatggata actcattcct caaaagcaag tattgatgtc agtcaaagtg 18960
tcagtgtaag atgaaaagtc ctgacagcat tgtttatgaa caagatagtc aaatggcttt 19020
gtcatgtttt catggtttca cagtttacac taagtttttt tccaatgcaa taaagtcaga 19080
ttttggtaac acttgctgaa aatgctcaag acaggactat atactattgg cacagccatt 19140
tgaaaactac agtaaagtta agacatccct gcatttaatg agggatttga gtacaagaca 19200
ctgaatatgt aagtttcaca ttttactgca tttgcatttt gcaatactaa tttagtcaca 19260
gcattgtgca gaagaataaa cacaccccta tttacaacaa acataaattt cctttgggta 19320
gagctgtgca caactggaaa caatattgca gtacatatta caactgaacc tacaacatac 19380
atggtgtagg tctgtaaatc attcatcaaa ttgttcaatt tagaagacat tttcaggatt 19440
tttttttttt ttttttactt tttaaaaaaa gatttaattt acatttgaca gattttgaca 19500
tctagttcaa aatttttgca tgtaatgact ccagtaatga aatgaggact attagtttta 19560
tataaaatga ctattcagca tcaattaatt ttgactgaca tgacattagc aaattaaaat 19620
aatataaaac agcagagagt tgtatgaaag caattaatgc atgtcaaaaa gcatctgcag 19680
tttattggaa ggaataagaa actgctatta tgatgtgcac aaatgaacta attgctttga 19740
gaaatgcatg aactaactgt tgtgcaaatg taaatagtgt tgggagaaat gcactaaagc 19800
gactgagaaa aactgtaatc taaaataatt taaggtaatg caaaaactat tttaaaatac 19860
ttcatattat attataatgc tctaaattaa attaactaaa acaaagcatc taagaaaata 19920
tactttgtat ttgaaaaaaa agtcaaaatg aataaatgta tatatgtata tatatatata 19980
tatatatata tatgtgtgtg tgtgtgtgtg nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 20040
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 20100
gacggacgga cggacggacg gacggacgga cggacggacg gacggacgga cggacagaca 20160
gatagataga cagatagata gacagacaga cagacagata gacagacaga tagatagata 20220

gatagacaga cagacagaca gacagacaga cagatagata gacagagacg gacagacaga 20280
cagacagaca gacagataga tagatagata gatagataga tagacagaga cggacagaca 20340
gacagacaga cagacagaca gacagacaga cagatagcat agatagatag atagatagat 20400
agatagatag atagatagat agatagatag atggatagat ggatagatgg atatatggat 20460
atatggatag acagatggtc agacggaggg acggaaaaac taatagacag acagaaatgt 20520
agatagataa atagatagac agacagacag accaaggata ttagagatag atcaaagaga 20580
ttaaagatag acagataata taatataata taatataata taatataata taatataata 20640
tgaacaagat agttgtcaac aagctactaa ctatgatttg aacatgatgt gtatcaaatc 20700
aaatgtcctg agctcaatgt cagcaggctg tgtgtggttt gatgctccag tgatcaaggc 20760
ccatttcagt ggcgttttca gctctggctg cagatagcaa ggtcagcaga tgtattataa 20820
agcaggtgcg cccacacaca cttgcacaca aacacacacg cacacacaga actccaaaca 20880
ctgccacatt gatttttttt ccagagggta ataacatcat cccggtattg tgaggagtaa 20940
tttaaactta atatgggtaa atactctcca atagctgata gttcatttaa agcttgacat 21000
aatgctgttt gtgtgcagga gaatgaagga taaagaatgg cagggaaatg gattaagcgc 21060
tgatgggcca cacagttagt caggcagctg tcttcattac agactatggg actaaaccct 21120
aagcaccccc taaggacgga gcttcagagg aaatctatag ctaaatgcat catcagagag 21180
acaaacaaac gcttattgcc aacactactg ctacatgtgc ctgcgacctg ctccgaaaag 21240
gagattctgc ttgttgttta aacagatgaa gtaaaggaaa cgcacctgga tccctcctcc 21300
ctaaagagcc attcaaaacg gcagatgtgt ttggtgtaaa tagaaggatt ttttttttagg 21360
atgtgtgggt gcttccaaca acatcaacaa aaaaagtgga atcatacata caagatgcta 21420
tgcaaatcat actaatagaa gttaaaaatg taaacatttt gataaaaaac aatttttcaa 21480
atgtgtttta aaaaaaaaaa agttatggcc aaatttagaa ttcattcacc ccagagtgga 21540
tgaaaatatg cccaacagca cataagggtt aacgtaagtt gcaaacaatg tatataggct 21600
gaatgtaaat taaatgtaga cattaaattg gaaattaaat gttgtaatgt tcaactaaat 21660
ttctttgttt aaattcagcc ccagctgatc tcacgggaaa acagaagtat tttacgtttt 21720
gccagttaat tcagtcgtac gaaattgtac gattttaaaa aggaggctcg gcacctaacc 21780
ccacccctaa acgcaaccgt cactggggga tgaggaaatc gtactaaatt gtacaaatta 21840
gatcgtacga attcttacga attatccact aaatcaaaaa gttacaaatt gccgtgagat 21900
tgtgttgatc agcccataga aattgtttgc aaccacttac cttaaaaatt tagtaaatcc 21960
aatgattcat tttttagtgc atgtaacata cttggttttc aagattgttc tctgttaatt 22020
tgcaagaaat gctctttaca caaatagaac ttccccatac ccctcaggta agatggacct 22080
ttttaggttc tcagtcacaa aaaacctccc agaaagattt tatgaaggtt ccttttttgtt 22140
caccagaaaa gtatacccaa agaacgctct caaaatgttc caatttggtt ccctaaaaat 22200
aagaaaacag gtaccaaatg gtaccttctg ggaaatgttc tgttgcctgg gttcaaagga 22260
aatcccacag ccactctttt ttctcacagc gtatcatcgt tctctaccac taatcaaaca 22320
aggtcacaca tctccagttt ggagggcagc tcggctctgt cttccgcagg gaatcgtgtg 22380
ccctgttctc cagagaaaga ggacacggag acaatgtcac agcagaaaac agccacaatc 22440
atcgctcctc cagggcttta cagtaacggc caaagtcaca caaggtgagt cgtcaaaggc 22500
agacgcacga ggtgaagccc tcaaccatct ggacaacaag atgcagctcc acacattgcc 22560
atgttctaca tatgtttatt taaatcacag gaagtggctt tcacttttgt aaacttcgaa 22620
gtctgaatat gcatgcacag aggccactgc ttcatattac tgtaacggat ttctgcatca 22680
tgtttacaga caactcaagt tcataggaaa ttacagcaaa gaaacagtct gattgacgtc 22740
tgaacttttt tgagcttcgt ttgtttagtc tacaaatgcg cattatatga cttgtgagag 22800
tggttctgat ttcgctatat gaaacaagat atactgtatg cataatgaac taaactccaa 22860
aaataacttc aaaccaagtt caaactactt atttgaaatt attagagttg aaaacacaat 22920
tcttacattg tttcttaaaa caactttgtt caaccaaaga ctatataata tacagtcaag 22980
acgtgtcact cgtaaagttt tgaacgggta aaattgtgac ggtcaatatg acgaatgaag 23040
cactgccttt tagtacaaga gccaatcatt caattcaatt tatctttatt tctatagcgc 23100
ttttacagtg aagattggta aaaagcagca taatatagaa gttctagcaa actgaaaatg 23160
ttagtccagt tttcagtttg gttcggttca gtttggttta attttcactt tcaaaagtcc 23220
aaaatactga agggcaaatc catcgatgtg cagctccact acttacaaac caagcaagcc 23280
agtggcgaga aacaaacttc accaattgac aaaagtgtag agagaaaaaa aaactcaaga 23340
gaaaccaggc tcagttgggc aagtacagtt ttctggccaa acttcatgtg cattactgca 23400
gtctaggcac cagagggtgg agaatgcagg atgtggagaa agaagagacg tggagaagct 23460
gcaggtgcga cggctgttta ggctggccac aagattgatg cacagactcg tctgtcactg 23520
gagtttttca ggactcagtc ctatgctctc cacttcttta tgactgctcc agaatctgct 23580
caggatatgg cctggtcaag gattatggag acctctagaa gtcctctatg gttggaatca 23640
tctcctctga ggtctcaaaa gatctcaaga aaagttatca ttgatcgcta tagatcgatg 23700

attctccagg aaaggggctt ggtttcaaca gatcttgtgt gattcgaaca tttataaata 23760
aactaaatgc agcgatgccc aaactttttc ttatgaaggg ccataaacct tgattgggca 23820
gaaggcaaat atagtttcca tgtgtaattt ccttatttat ttaaaatgat ttaaaaatgt 23880
ctagactaca ttgctttata tgaacttata cagtattttt aacattttac aacgaactta 23940
ttacactaaa tatctaattt ttgccttgat ttgctcgtcg atgtcttctg catagtccgt 24000
cgagatcgta cgaaaggtcc gtcatttaaa tttttaaaa tctgattcat ttacaacatt 24060
taatttaagg atttaatttc agtttgcttt ttttgtagct cagcaacaaa acaaacaaat 24120
taaaactgca agcagcaata aaagggacct tgcactatca aaaaaggtta cgtcaaatta 24180
gaaataatga tctcggtgtt aaaagcattt gccccaaccc tctccatcat tctcactctc 24240
atctcagttg gggtggtgaa ccaaatcaaa ggttacaacg ggccaacttt ggcctgcagg 24300
cccaactttg agcatctctg atctaaaaaa acagtcgttg tttaatatta ttggagattc 24360
ctaatatgaa atgtaagctt ggcatgcaat tttagagaat ttgttgtttc cccatttaaa 24420
caaaatgccc gagaggcgtt tcaaagacgg ccgctgagtg aaatgactag ccttaaaggg 24480
actctgcttc aatccacttc agtttgtaaa aaaaatttta tcgattcttt ttgagacaac 24540
atgaagaaat tgtgcacaac ccagcatttt tacagtgtag gtcaaaataa gacaaacctt 24600
cagcatcttt catcgctgcg atgcagttag ctagtctgac actggaggta aaaatgcaag 24660
atattttttc tttcgtatta catacagatg tttcaccgga taaaatctgc ataacgttgc 24720
caagagcaat tattagtttt atgcagtcct tctgagctga attaattaat tacaagtaac 24780
aagtcaggtg tgaactgtcc aatcacatca aaacaaactg ctttcacgct tgaaagagaa 24840
tgagattttc ttaaatactt taatgaggtg ttaacaaaat aagtggtaca gtttattatt 24900
ccagtcagtt gatagtgttt ttcagtgtgc atattaatac acaaaacata cagagagaag 24960
aaaaaaacag gtccaattca gccccccgat gcttctttaa agcatattag cacatcattt 25020
tctttttct gggaatgcat cgagggcctt aagcgattca atcatttcta acgccgggtt 25080
gctaacgaga tcctcgcagc tccaaaccgg tgtgaacatc tgtgaaatag taagcgagtt 25140
gaagcgtgca tggagtggaa tgagagaggg tgatgctgca tacgagtagc ctgtttaatg 25200
tcatcatttg gtcattatgg tcaaaggtcc tttcagttta gtccacaaac aaaaaacacg 25260
tccagacatg atgtttaaaa aaaacgcatc accccaaaaa ccgggcgccg tgtggagtaa 25320
acacaaacct catcatgcat ctgatcaggc ttgaaatccc caaaagggct gaaagaagcc 25380
actttacggt gactgtatcg aaaaacaatg ctcaccgatg ctcagtttgt tcaatgcaaa 25440
aatacatcaa cggaatatca taaccagaaa ggcaatatat aacatttaac acaagaaaaa 25500
gagcaagaaa tcaaacaaga aagcgagaaa tagtttatac atgctgcagt tagagtagta 25560
acatgcgttt acctcgtctg tttggagatc gccaagagga acactcattt tagtgtctta 25620
ttttggtcct gcattgaaga aagctgttgt gttttcacct ctctatacag aaaatgtctc 25680
ctccattcac agttgtaagt atctacaaca gtgttcaata atgttccctg ctccaatcaa 25740
tcttcaatct aaaacgagga aacaagtcca accgtcttaa aacaacttct ggaaaactct 25800
gattcaacac attgtgcaca ttctatatag atgacagtac tcttaaataa aaatatagta 25860
ataaaacaaa gtgttctcat ccttacgcta cagaaaacag ataatcagaa actttcttca 25920
agaatatccc atgtggtata gagttttaaa atggcttact atatatgctc aaaggattct 25980
ctttcatatt tacaaaataa aatgcatatc tttagcattg ggaggtcctg cgtaccatca 26040
cagtcattct tttttccttt agtttgcaga gtccggaaag ctaaaactgt tggtcgaagg 26100
cactgacaaa tgtcctttca tcagtgctgc tgattaacat gatacaaaaa taattgagaa 26160
aaaaatagac acttaaggac tagtggttac aaatggcaga aagtgaaaga catttggcaa 26220
agttactgtt tcatctggtc ccaacaaagc aaagtcaggt tcttgttgct tcctttcgtg 26280
tgattgattg cgaccatgca aataaaggca gcgatgatat gcgagtaaca gagggataaa 26340
gcgaccgttc acacacactc ctccgcagac atcagcagag ggttgatgta ctcgaagcct 26400
tcaaactcag actggtcgat cttctttaca gcatcactgg gggaaaggca aacacataat 26460
caatcagtct taacccttta ataggcatcg taactgtctg cttgaccttt ggtccactga 26520
ctttcattca aataaaaacc tcaaaatcag cttagagtgg tggtaggaac aaaatttcaa 26580
taaaaaattc ttagtgtgaa atttaaagag gccaaacaat gttgatatct gaaagcaatt 26640
ttttgatttc tccactaaag ttcctataga cacatctctt ggtgctgatt ggctgcaagt 26700
ctgttttggg acttggtcat tttgcatgta aagtcgaaat actctcactg acgcctctgc 26760
atgagttaat tttttatgtg accattactt agaatgcatc acttcaactt ttccatggca 26820
atgccacaac accagattta tgtatatccc attttatgta tattctgcaa atattgacaa 26880
acatcctaat tttattgaga aatatctgat atgccaatac taaaatatgt gtaaggaaat 26940
ggactaattt aaatgtctaa gacataaaat gaatgtaagt tgtctgaaaa caccaacaat 27000
attagattta tgacattatt atccagcagc tgatcatgtg acgcactgac tattaatcac 27060
accagtgtga tcgtgcgcat caaagagtta accactattc tcaaaaagag acaggctata 27120
aaatatcttc cagaatgttt atgtgtaagc acacagactt tattattcaa aaaccttaat 27180

aaagatcata ttgatggggg atcagagctg ctggatttaa aacatggtaa ttcatcatct 27240
tcacccaaat tacatgaaag taacttactg tttagctgag aaatgaacag agtgaaggtt 27300
attgcacgat gaatccaaaa ttttcgcctg taatttttgc acattaaaaa ataaattcaa 27360
cctcacgttg tgtctatcct gttgacagac tgcctctgaa actttcgtcc gttataaaaa 27420
aaaaaattta atcgggtttg atttttaat ttttcacgtc caaaaagacg ttttgagagg 27480
cgttttgaca gttcagagcc accatacaag caaaaggcaa aacacaaaat cccccttactt 27540
gagccacaga taactttatg acaaacacag tgcatgatat aagacaaaac atgtcaaaga 27600
gttgaaaacc catatgtctg aatcagtgac tagttcactt ctaacctgct gctgtttggg 27660
ctgtgtgtgt ctctgtgtat gtgtccatac atttgacgta ctgtccatag acattataat 27720
agaaagacac ctcatgtttt ttttcggtcc gttgctccaa tgtcaatgtg gccgccattt 27780
attctaatgt ctatggtact gcaagtctct gttcagtcta tgtgaagcat cacaagcaat 27840
gtatcaaaat tccactgatt ttctaaaata agtgttgcat ttacattcct ctttcttttt 27900
ctacttcaga aaagagagga gaataaagta tcgctgcttg aattgactcc gaaatgtttt 27960
gcattttttt actgaatgga ttaattaaca cgcatcggac tcagtatgca agcattgtat 28020
ttgttcgtct tacacacgag acgttaaacc gaggtcctga ctctttgtgg ttaaaaatct 28080
catgacattt cttgtaaaga gcaggggtgt aaccccggtg tcctggtcaa attttcttca 28140
tcggccctta cccatcatgt actcccaatc atccccatcc aatgaattgg ctttatcact 28200
atctctccac tccaccaata gcttgtgtac aataaataaa tgcatacgta aattttggac 28260
ttcagtgtgc aaagacctta aacgttttag taacattaac agtgtgacac acatcgtcag 28320
ataatgtgta acatgattat catagactat cataaacatt agcgtgacgt tttacaaacc 28380
aataaatgtt atgttttgct agcacttggg taaattaagg ctgaacatta tatcgtttaa 28440
gcatcgatat cgcaatgtgt gtatctgcaa tagtcacata gcaggattag attattttta 28500
agattaaact ataaggatat tattacattt tattgttatt aaaactgttt atacaataat 28560
aagcatgttg ttttacattt gactgttcga tttctgtaca tgaatactct tagacttaac 28620
aaaaaatcat aaagtatcgt ttatccattt gcttttgctt gtaattttta ataatttatg 28680
cagatccact gcattaaatc atcccagtca atctacattt ccaaatagag ctaataaatt 28740
catctggtaa aattatattc atatcacaat atatattgca gtaaaataaa atatcacaat 28800
gtcagatttt tccagtatca tgcagcccta atttaaatga ctgaaaccta aaattaatgt 28860
catttttctg aaaacaccaa caatgttaca tttatgatat tattattcag cagctgatta 28920
tgtgacacat tgatcatttt aatcacacca gtgtgatcgt gcgcatcaaa gagttagcca 28980
ctattttcaa acagagacag gctgtaaaat atcttccagg aaaactgggt tatgtggaaa 29040
tgagtgaatc gagtctgtaa tgtgtcatca tacatcgtaa aaagcaatat tacttcctga 29100
tgaaagctgc attgctgtgg tgaagatgtg aagtagatca atggcgtctg agcctgtgat 29160
tcagaaagca agaggcgtga cagtgacaga tgtacatgaa cagacactca ctcatcgtca 29220
ggcgtgagct gaatgggctc gttggtgaac tgggcatcaa agttatccag accaaactcg 29280
cccgagatgt taggtttgaa cggtggaact acttgcttct gctccatctg aaatacaggt 29340
aacatgagtc aaataatgac tgaaatatac tcatcataat gaggttacta cttcttaaag 29400
ttaattaacc attaactata aagaacagat gtttcagtta aggaagcaag aattctacat 29460
gtacatgagc caaaaatgac gtatatttta accatgattt actaaagaca atcatgataa 29520
gatcattcag tgtagcaata gtttatacac ctaaaaaatc ctgtcatatt aagaacaaga 29580
gtcactggaa gacatattaa aaggttcagc ccaaaaatac tactttagat acctcatcct 29640
ttgtaattat ccttccagcc actatatttt accctttgt tagtgggttt ttaattcaat 29700
acaaatgtac tacatttaaa tattctttta tatatgtaac aaatatatgt tcacttattc 29760
ctttatgtaa tacatgagaa agaacaacat taagcatttt cattttgtct gtttatgcta 29820
aaataacaca caaatttgtc aattgtgctt gacataaatc tttttgaaga ataaaaatga 29880
gtgataataa ttctctgata ttttttattt tgttactttg agcaaaaact taatactgag 29940
ttttttaggg ctgctcttcc cagctcttac tctgtccttg ctgtaaggtc aggattagct 30000
ccccctgcag gccaccacaa acaccgcagt ctgtctgaga gcaaagtgta atctgatcgt 30060
tcaaactgtc tgaccgctac agctgtctgt gtgtgtgttg cccagacaaa acagggacaa 30120
gaggtcactg tttgtctgag tctcatctgc tggtttgaca tcatttaaac ctgcctcaca 30180
tgatacagtt cacagtctca accctaagag cagaaaaatc aataacatga caccttatca 30240
ccttcattgt atcactgccc ttacacgtga tcatgataca accacaataa aataaaacac 30300
tcagacttta atacattcta cagattcaga tatagtatac caatatgtat tagtacggaa 30360
actcgacaga aatcaaaaaa ataaattata aaccgcttga ggatttgctg aatttaatat 30420
ccattggtta aagtaaaaca ctaatattgg tttaattcta aaaggtctg aaaaaaagtc 30480
tcaatttcaa cttattttgt ctctataata aacactttca tttgttgtga ttgaaatgca 30540
gggttgttat cccatacatt attgatctct tgactttttt acagttaaaa cattgtcatt 30600
gtgttgtaaa ataactaaat ctaaaaacat gcaattttg ttatttaatg ctaaaaaagt 30660

attttaaatt tgataaaaat ctgtgcaaat taaacaaaaa atgtaatctg gaggatttta 30720
aacgctaatg ctgtctaaac taatgctcaa gacacaaaaa acctcaaaaa agtactccag 30780
ggtgtcttaa aacctttaat tttgacgcca caaataagtt cttgactttt aaatctaaca 30840
tatgtaatct taaaatacac ccctcacttt tctgtttgtg taaaaaaaaa atatacacaa 30900
aactaatagg atgtgtttat aatgactcat ttttgggcga gctttacttt caaagctgac 30960
gctgctgaaa tgctacttga agtggtaaga gctcacgacc aaattacagg aatgactaat 31020
caaatgaagg attcctcaag gtcataaaca acagtaaaca gctcttcagg ctccagtaag 31080
tcctctgttt agtttcacta acgtcaggct agagagaatg actcattgct tcctctgcgt 31140
ctcaagtcaa aacatccaag aagacagata aaggaaacgg agataacaat gactcacaag 31200
atcccagtct acatttcgga aaaaaggatg ggccatgatg tctgcgaagc ctgtctgagg 31260
atgacatccc agccgttcct tcgactccta caaaaaaaag gactaaatgt cagtgagaag 31320
aaataagata tattttagtt ctttttgct ctaattgtcc agctaattgt ttgctaaaag 31380
tctaatttga tattcatctt gggcctcatg tatcaacgct gcgtacgcac aaaaactttg 31440
cgtacgccag gattcacgct cagaatcgct cacgtttgga tttactaaca atgaactgaa 31500
cgtgggaatg tgcgcaggtt cacggcagct ttctggctgg cgtacgcaca tttttgtgc 31560
gtgtctgttt tatttccatt ggcgactcct agaggcagtt gtgttaaatt cctctctaca 31620
aagtgtctga gccttgcaat ggcagctgta tgagacgggt tcatctagta ggtatacaag 31680
gtttccatac catacagttg accagctaaa cattaaagca caatttgcag cggtcgcctg 31740
ttttcccaat gtaatctgag cgatctaccg cacgcacatt gctataaaga cactatctga 31800
agatgaattt gcatgagtga atcagaaaca tttccattca attaatgtgc aaataaaata 31860
tgatgcacaa acttattgat gattcctact tgtctttctc gtgataaata gtgggcaaaa 31920
tctgatatgt agcggggaaa aaagaagaaa gagttcatca gacgctggat tcgagccgag 31980
tttatgctcg aacatgtcag tacatgatca catgcgtctt acgaggtgcg ccactgagac 32040
tgttaagggt actacaacat tttacagata taaaccacac tatttatttt ttaaatgcac 32100
tcagtgcgat gttcagaccc aactgtgtta accgcatcag ctaaactctc ccactctatt 32160
ttttttcttt tgttgttaat tccggagaac aaacttgcaa ataacaccgc ttttctccgg 32220
tctacctccg aaagcagcac ctccatttca cattctgttc aaagtttctc tttttgcttg 32280
cttttgccat tgcttttttg ttgggttttt gcattagcat agtcattagc atattcatac 32340
gggggaagag gcagggaggg gttttgtgct cgtgcatgtt gcgctcagtt tcacgttcat 32400
tcggatgtac aaaagaatat gcgtgagatt cggcgtacgc agtgtttcat acatctgaat 32460
ttttctgcgt acgcacattt acagctttgt gcatacgcaa tgttttagta agatttccac 32520
gcaagtcttc gtacatgagg cccctggtct ttgtatgaat aaaacagaaa cagtattatg 32580
tacaatatta gactaaaaag gaggatcttc atattgctgc ttttctggta atttaacttg 32640
cttctattga cctcatttgt gagttgcttt gaaaaaaaca tctgctaaat caataagtgt 32700
aaatgcaaag agatgaacag acacacagaa tgattaacac acaacagaag gaaataaagt 32760
aaagaatgat tatggcagca ctataacaag ctcatctgac gtaaggacat tagcattgat 32820
ctgtctggtc tgcttcacct tgttgaggaa tcccttcagc acgcttgcgg ctttgaccga 32880
taacgatctg ggaattctga tctgcttctc caaaatgact gcagaagaag tgtaaaggaa 32940
aacatgagac acgcacagaa agacaaagca tttttgaaac gttcatgctg ccagtcatgt 33000
accttggaaa agataatcct ctgtgttttg gtcagggtta tcagagctgc cgactatgtc 33060
gaagggagat cttccagcca tcatctcaaa catcaggacg cccagagccc accagtccac 33120
actaaaacct ggacacaaat aacatgcatc attctaaggc ttcatactgg tataaatgta 33180
atcgtcagtg ggggttatga agcaatcgct aactataata aaggtttcaa aggtgcagta 33240
tgtaggattg attgaactag gtattgcagt ccaaattcaa aatattgaag tttgtttttt 33300
ctcacttagc ccttccgctg acacaaaggt tgccagattg attacaacaa caggagaaag 33360
agtgccttca gtttagcctt tcactgtaaa cggatcagct aatgtttatg tttgtagttt 33420
tttgctaaat aaaaactagc tcatgtagat atacaggtca tattttggca gctcagagag 33480
ccaaaatata atgagtaaac ttgcagtggg gggagttaca gagaccaaaa cattgacaca 33540
aaactcccat tttcaaaagg agaataactg actctagcat catttttcag ataaacaagt 33600
atgttcatta agtattcatc ttaaatgtct gcatatataa catatggtat ttttaagact 33660
cagaagagcc ataaacttac atgcagcacc ttcatgcata agcaaatttg tgattacagc 33720
gttagcaaac atagttgcta actgaaaatc ttcataaaga gtctctgctg tgtgtgaatt 33780
gagtttacca tagtcttctc ctctcagaat ctctggtgca atgtaattgg gagttccaca 33840
gaaagtgctg gttgtatctc ctggtctcag tccctcctgt gtaacagata acaataatga 33900
aaccaaaaaa acttataaca cacattagct atgtttccat gcacctattt ttatgcacat 33960
tttggataca agcaaaaaaa aattttgatt aatggaaatg tcaagaagcg catcaatttt 34020
aaaacgagta ggacaaactt tttattttat aataaaacta aactaggatg gaaacacttt 34080
taccgaacaa attccaggtt gcgcattaaa actggtcatg tgattttgtt atgagatcat 34140

gtaatgaaaa aaatatgtgc gaatggaaaa accagcaggc tgagctcact ataaaacatc 34200
tgaaatgttg ttttagtcat tctaaaacgc cttaaccgtt tcagcattag tgtcattata 34260
tcattaatct tcgcgtttca cgcctttaaa cgccaccaca tgttcactgc gagtcagttt 34320
ttatgatcat ttgtgttttt gtttttgggt agattaaaag ctttcattat ttttaatata 34380
gatgcttttt aatacatcaa tgcactatat tatctaaatg atactttatt tcattttaaa 34440
ttattaattt aatttaattt ataaatgtac aggtttatac ttcctaaaat catatcattt 34500
ttttcattaa acaaaaaggt tgcagtaata agaagtttat taccagcagt gcaccagtat 34560
tttattcaaa agcaggtgag gtttgtgaca ttaaatgtta ttagatgtca gacagtacat 34620
ctttttctaa aactttgagt ttgtttccac tgctgaaaaa agaaaagcag tgcccagtat 34680
aaacgaatgc actctttaaa gatttctctt aaaaatgaat accatctcag accccctagt 34740
tttttcaaga tttcacgatc gctaaatcca acacaaaatc aagaaaaatt cgtaaatttt 34800
agtgatcctg caaaactctt aattaaacgc aaaatgattg caaaataaat aaataaataa 34860
ataaatatat atatatatat atatatatat atatatatat atatatatat atatatatat 34920
atacatatac atatatatat atatatatat atacatatat acatatctac atatatatat 34980
atacacatat atatatatat atacatctta aaacatcaaa ttccanacca tataatcaca 35040
ttatatttat tttcagttta ttattaattg tattacgtga ctaatagatg ttgttgcatg 35100
cgcagcgcac atcatgtatt tgaggttctc ttgaaaaatg acgtttcacc tgcgtcctta 35160
caatcattac attatatgag agtaggcaga attgttttgc agcctgtgca gtaagcagac 35220
acggatgaag cgccagtgat ttacatttga agtcaatgca aagactagat taggcatcct 35280
gtggcacgaa ttagtctgtg catttaatgc acttcagact gaaaaaaaaa aaaaaaagag 35340
caaacatcta acaaacagag caatggaaca gacagaagaa caagcagctt acaaagatgg 35400
aggttgattc aaggatgacg gccgctggac gtgactgaat tgaaggacat tatttgtgct 35460
ttacatctat ggctggtaat acgatgtgaa taaaatcgat aaatgttttt gtttgactct 35520
tttcctgcag gtaccgagag aaaacgcttc cttgccattg atgagtttta cgttaatctg 35580
tttttaaggt gtattacagt aggggaaacc ctaatgtatg tcctgagtga ggtacacaat 35640
gtcagatgct ccggagagta aaatctgaga gaaagtaaga tcatggataa aaataagagt 35700
tatacaacct tcctttgact taatccttac tgttttagat cttgtcttga caagagacca 35760
aaataaagaa gctttatttt tatgatttgt gtcattcttg tgtcaatcat tgtcagattg 35820
cacacctaac aaagtaggca acctaatatg gtaaatatat ataaatttat aaaaataact 35880
atttttttat tgacaaattt ttcctaaaat ttctgggaat taccaccaca aaatatatat 35940
attttctaaa aaaagtgaaa actagggggt ctgctattga aaaggattct atacaataag 36000
atatttgtgt ccatacatta cagtagtcag tactaaagcc aaaacttact taagaaaata 36060
ccttaagatt gatgggcaaa aataagtacc aactaatgaa tatcttaagg aaaatgtaaa 36120
ataaatcttt aatacagaaa aatcaagaga accacaaaaa tatagaaaat tgtattgaaa 36180
tttttcagtt tgtcattttt ttgacaacat taaatttaaa tgcatttttt attcctaaaa 36240
atgtcaggtg atcacactct tattttaata aatatttaat aactgtatta aatatttaac 36300
tgttttgttt aaatggacca aaatacatac attttcaata tccacaagga catgtataaa 36360
aattcatttt ctaaaggtgt tgtactcatt caaaggttac catcaaaatt ttggtaccca 36420
acttttttca caattacttt ctgtttggag caagtggaaa atttaaccaa tgaccacatt 36480
cagacttacc ttacacatgc cgtaatcggt gagtttgatg tgtccctctg aatccagcag 36540
aacattgtcc agtttcaggt ccctgtaaat aatgccacgc tcatggaggt agttcaaggc 36600
aagactgatc tctgcagagt aaaacctgcg agcggaaaga acaagacaca cagtaagtga 36660
cctcattttt gtttctgttc ctgtggcgag gcgttcagac aacgttaaaa gtgttatgca 36720
ttggatgtcc tcagacctgg cgtgctcttc cggaagtttc ctctgccgct gcatgtggaa 36780
catgagatcc cctccattca catactcgat tacaaagaac agtctgtgaa agaacagcat 36840
taaataatca ttagccccta ttatcagaca aagacacagt tcacacttaa ctaaaggct 36900
caacaataaa tggcctaaag ggcacttata ttgaattttg ggtgtncact ttaaaaaaaa 36960
aaaattttac tttttattct cactacatct tacacttttt atacatgtat tttaatgatg 37020
tcttatgact aggctcacat ggaatctgcg tacgcagaac tctgcagatt ttccgcagat 37080
ttttagccca tcattaattc tgttcattta cccgagtaaa tgtgtgtaaa tctatattta 37140
tttagttttt taattaaata aagtaatatt attgactaat gtaaaaatgt tcatcttatt 37200
tatgtacaat gcagttcgta aagtattatt ttctgtcttt tagtagatat attagatgag 37260
agacttgctt tgtttaccaa ataaagtgaa tctaattgga tttgcatttt aaacattaaa 37320
tcaaaggtaa aaagatataa ttttttattt tcacattaag gttttagtta taatactccc 37380
aaaatcattc cgcagaaatc cgtagatttt taccaaaatt ctctgcaaaa atagcagaaa 37440
atgttagcag attcagtgtg gcccttctta tgactaaatg aacattgatg tggcaaaaaa 37500
aaaacactct tcaaggctgc atttatttca gttgtagtaa tagtattttt tcatctttaa 37560
tgcatttgta aatgtcattt tttcctatga tgacaaggca gaatttccaa cagccattaa 37620

atccagtatt aagtgtcaca aggagtttca caaataatcc tattatgctg aaccatatcc 37680
tatggtgcag cacaagtgta tttcttattt tcaacaataa aaaccgttta gttgcttaat 37740
attttagaga aagctgggat gaacttatga ccatgaatgt tgttctaaaa tatgcttgtg 37800
aaaaatatta taaaaagatt ttcagaagta taaatctgta aaattgacta aaaataggaa 37860
taaattaatt tgaataaaaa tatttttttg ctaattagta atattcacaa tattatattc 37920
gtttttacaa tgacaatcaa gaaacaaaaa cacagtttac acgaatttca taatataaaa 37980
ggaaatgaaa tacaatctgt atcatatcat cagtaaatac aaatacacaa attataatta 38040
aatacattac aatactttga aaaatataaa ggcgggtaaa acaatctaac ttaaacataa 38100
tataaactaa ttctaaatta acttactgac tttaactaaa ttattagctc tttttaatg 38160
gaatgctttt aattaactat agctaattat taatttaatg accatggaat gactacaccc 38220
cagtcgaaaa ttatttttga acatgtctca ttgcttttag ctatatattt tggtacattt 38280
aaacaaaacc atttattaa ataataattt aaaaaaagag tgtgcttacc taacatcttt 38340
aggaataaaa tatgcttgtg aaaaatatga tattaatatt cacaagtata aatatatata 38400
agtgtaaatt aataaattaa tgtcaataaa actattattt tgctgattta gtaatattca 38460
caatattaat gagtttaacg atgacaatct agaaatgaaa atacagttta caaacgtatt 38520
caattaattt agtgtcatat tataagatga aattaaatat tatgcattta aatacatctg 38580
tattacaaaa ctataaaaac ttttgatcta cacaaataca aaaacacata ataataaaat 38640
acgttacaat actttgaaaa atatgaagac gggtaaaaca atctaactaa aacatagtat 38700
aaaataattc agttcaaata tatagcatct catttaaaaa aaaaaaagtg ttaataaatc 38760
aataattatt cagtcatagt taaagatgct gtttgtaagt tgttgactca ttttgaagca 38820
taaaaaaaaa taaaatgttt gcagatattt aagaaaatgc caagtaaaca ttcttgttta 38880
tctgaaaaac aatgctgaag tcagatattc tgcttaaaaa tgtgagttac gtgccggaac 38940
gcctgtcttt gttttgctcc tataacccgc ccaatgccag atgagccaat aacattccag 39000
caccctgggt tgccttggtg gaaaaccgca tatttcattc attcattcag aaaggatctc 39060
aaagcatgcg cccgtgaccg aaatgcgacc tccggtggac agtagaagac tccgaaatga 39120
gacgcagatt cagagttcta tatgaggtgg ttattaatta gcaaataata taaacactac 39180
gaccataaac attaggtgag cagattacat tgtaacccta tggtccaaca acccactacg 39240
tgaagagatt tgcaaagata agcaatttgg ctctttgcac aagacaaaca cgacagaaat 39300
ttaaatacag ccattcagaa acacagaata tgcactcaca caagaaatgg taaggtttat 39360
catctaatta atacatatta aacctcttta acattattaa atgtagaagc tgagtcattg 39420
acatgtgttg gttttgagtc acagttcaaa gttcaatttc aagcggctta ttttattttc 39480
gagatctgag gtgaacaatc tgctgctgct ttcaatagta tggcaataaa tgtcatttaa 39540
aatggtattc aaattcacat tattagtatt taacactaaa taaagcacat gaggtgtacc 39600
tgaggagatc actgtcagct ttcagttgtt cagctatgag aaatagaagt tgtttctaat 39660
atatcaattc aaggtttggt taggacaaaa acttttaaca ttgtaaatat tccttctatt 39720
gcgtgccgtt tctttcatat acaacacttg ctcctctttg tctttaatct ggcaacatgc 39780
gtttgcattt gttttgatcc aggaatgcat tacctagttc aaccacttgg tgtcaaactt 39840
acaaactgca cctttaatca aaggtgttcc ataaaaaagg gctaataatt tagttaaaga 39900
aaacaattga gagcttaact gcataaattg taaaatagaa ttaatatgaa cccatgttta 39960
aaacaatcgt gaataacata acaatatatt tgatttgatg atctaaaaat gtgtgcagag 40020
atggacagac ctgctctccg tctggaagca ggagtgaagt cccacaagga agggatggtt 40080
tgaagcctgc tcaaacacat gcttttcagt ctgaacccag tcaatatcct gaaaaacaag 40140
aaagaagaac aaacatcaca cacacacaca cacacacaca caaacacaaa cacacaaaat 40200
caatctccca ttgaaaagca gagcattctt tcagtcagta ggggaaacat ttggaaggac 40260
attcaggaca gtattgagca gaggctcctc gcaaggctga aaactgataa aggttttaaa 40320
agacgggggc accaggtctt cccgagaagc tgctcctgta tgtgaggaga ctcataatgg 40380
acagatacag agtgaaaaac taagaccttt cattgacagc ttggtaaaaa tccctctgac 40440
cgtgagactt gcataaagaa gcactgaggg gccagtagag cacagctgcc attattgacc 40500
atcggcttct gctgtcagtt catcacacaa gccttaagtg cggctaatca tcgttaaatg 40560
gtcacttcat caagaccctg tctgtttgtg tgcacttcaa cacattctgg aaagcttata 40620
tccaacgacg cctctttgct taatttattc agcatagtca tgacttattt tgcttttgtc 40680
aggagaaata ttttatttag taaatttcat gaagtctgtg tagaatgtgg tttatttgaa 40740
ccaattatga ttcacaatta gagctgtaaa tttcatttaa acggtagggg gccaagaaat 40800
aatacaatgt aaagcaatgc acagctaaac aatatgctaa ttgtggcctt taaaattata 40860
cccagtcgtc acaaatataa caatgcatgg gaaatggctt tggcatgtta gttgcagtgc 40920
actacgcatc acactatagt aaacaagcta atgttaacta gataaataat attttaatcg 40980
ctaatacagt ggattcaggg aaaactacat cagtaatcag catttttgca accaagttat 41040
gaacgagtct gcatcaactc tattgatgaa aatcgcttta ttttaagtga ctaaaatacc 41100

ctacttactg gagtccaaca taactaggc cccagccaca aacctgactc gtgtgtgtgt 41160
gtgtggtgtg tgtgtgtgtg tgtgtgtttt tgtgtgtgtg aattagtata gacgaactgg 41220
gaaagcatgc agtggtccaa accactgtga gcaaggggag ggggaactca acggtttata 41280
aacgcactct ttgtgtttgt tttcttactt atgcaattat tttaggtata catacatata 41340
tttttcacaa tagagagtaa gatttaaaaa aaaaatgttt tggggacaac cctcagatgg 41400
aagacatctc tccgtgtttg attttcttat acatacgatt ttcttatata cggttatgct 41460
gtcggactgt tgtataaacg caacatcaca cttttagcag tgtgatgcgg ctgtatattg 41520
tcacttgtgg gacacaaagg cattcgggct gtggactcgt gccaacgcac gcctcccacc 41580
agtgccgata tacagctaca tcgcactgct actcgtgtga tattgcttaa tataatttca 41640
acagatgact aaagattggc aattatttta gaaacaaatg taagtgtatt tgcttaaaat 41700
ataacaaaca aatttccagt aaagataaaa acaaaagatc aaaatttcaa aagaaataaa 41760
cactgctttt ctggttctgt tttccttctg tatagtctga ccatgcaaaa ctaaatgtaa 41820
ataaaatact gcacagtcat cactgtataa attaaataca attaatattt gtaaagctgc 41880
aaatgttggt gtttcttgtg catgaatgct ctcttgttgt gtttaagcct ttggttcaat 41940
ccaactttgg attgattttg cagacacaga caaattttca aattctgaga caaaaccctc 42000
cagattgcag ctaaatgagg aaatcgtaga tctctctctt ggctgtgact gcttcttgac 42060
caaatgcgct tttcccctgc atggtacatc ttggctcggc ctggttctgt tcggttcagt 42120
gcggcttgaa tcttctcgct tttctttttc actgcagttt gatatcgctt taattggtgc 42180
gattacagtc atatcatcat agctgtgccg tctgcactgc attgccatga cattctgaat 42240
cagcccattc agctctgaca ggcactcatt tatgatgctg tgacaatcat tactttcaat 42300
tttacattgt atgtcattat tgttcccctt gtgtgtgagc atgtgtgaat gtaaaaaagc 42360
gagaggaagc tttcgtcttg tgcagaagcg aaatcaccac ttgatttttt cgctgcttgt 42420
tgtctttaca ttttcatata gtttggcatg tatgtgtatt ttaggattat ttcggattaa 42480
agcccaatac tattctattt gtgtacccct accttttggc ccttgaaacg gagtgcaata 42540
tgtcattgcg agctcatatg agatcgatga tcgcactgct gtagttattc cagttgcact 42600
atattttggt atttatcttc aggaaatcgc caaaggcaat aatatcatgt tgtcataaca 42660
atataatgtg gcaataagct cgtaactgta ctgtgcattt acactgtggc catattcatc 42720
tatgcaaaag aaatcaacat taacgttcta ccagacactg taaaaaggtc attcccagcc 42780
gttagacttt tctgacaggg tattccgagt gtcatcaagc attagatgtg gaaagtatgc 42840
tgtgggacag ctatacagga gctattatta tggattctag attttatcgt ttattttttt 42900
aaaacatcat aaaacatgaa tgccattatg cttgttttaa cacaaaaaca cactcattta 42960
tgtattatca atgaattaag ccaggaacaa agatggcggc ttgcagttgg aacgtgcaaa 43020
cagtgctaat aatcactgcg ttttatagtt tgctttctcc agttaaggag cccattttac 43080
atttttaata caggacagtg atatggtgaa gagacatttt ggtaggagag caaatattgt 43140
ttaagagtcg tttatatcta ccgtgtacta agaaaaacaa acgtaaaaaa aaaaaaaccc 43200
aacaaagctg tgatggatag ggactaagcc gaaaaggaaa ttaatgatta aatatatata 43260
tatgtatttg tgaataattt tgttcctgtt ttaaaatgtg gaggtactgt ttattttata 43320
catcgactgt attttgaagc catactttat ttaccagcga ggcccacaat ggaaacaacc 43380
cctgggcttt attgtgtttt tatcatcaac agtttaatta aaaaatttag ggctacttc 43440
tatttttgta cagcttgtct aaaatctgtc aaataaaaat tcaatttaaa attcaattca 43500
attcaatctc ctgacttctg tgtgcagcca tgctgctgtt gtagctggtg tattccttgg 43560
aaattttcgt accccctcggt ttcgagtgtg gtcctggaaa atctctgttt caaggactat 43620
agtccttact cttagtccaa tgccttcaag ctaaagagaa ttgaggcagg gaagggaaag 43680
ggctaagggg ttgaattggg attagcctaa gtgcctggag tctttttatc tcacttcaat 43740
atagaagtga tttacaatat agaagacgtt tcacataaag aagtgataag tgagctgtag 43800
caaaccaaac tgtggcataa aattttctat gacaagaaaa aaaaacacaa catacaggta 43860
gttcacaagt tctctaatgc ttgcttttaa aggtcaggtg ttattcatta tccaaaatca 43920
tgctaatgat ataacagcgg cattgtttgc aagttcctat ttttatgaaa tgtgaaaagt 43980
cacctcgtca tcgttgacca gctccttctt caccaccttc atggcataga tgcgttctgt 44040
ctttttgaga cgcaccagca gaactttggc gtagctgccc ctgccaatca ctcgcagcag 44100
gtcaaagtct attagaccca aactggacac cgcttttcct gattcccgac tgcccacagc 44160
ctgaaggatg catgaacgga gtattaaacc agtatcatac aatcatacat ttctgcttcc 44220
tggtttagat gttcttctct gtcctctctc tttgtttttc ccttgagccg cttgatttcc 44280
tatgactgcc acagagctac agagctcagg cccttaatca gatgctaatg accctgatgg 44340
aaggttactt tattagctgt acgaaaggaa atcaatagag ccgaatggac cttgtgcaca 44400
cccccatacc actgcatgag tgacaagtcc agcctaatca tcagtctgag agcgactgct 44460
gaaagctcat ccaatcacac actggtcata atagccacat ttacacctgc aactagcttt 44520
tatttagact gatttcatga gcaggtggag agtgttgaag acaaaccgaa ttttacaaac 44580

cacatttaaa aacattaaat aagcaagcag tcatttaaag aaaaaaaaaa tacaattgta 44640
agtcaacatt aacaactatt gctattatta ttttattgtg gtttaaatta taaacatcca 44700
acttaaaaat aataaataaa aatcattgat attctaatat aaaaatgtct tgatattaat 44760
acaaatgtat tcttacaata ctaatattta tacaaacatt atattttccc aaaaactcat 44820
tttttatgat atatttccaa ataaatcttt atccaaaggg aagcattgaa gattagttgg 44880
tgttgtatac agaaatatac atacaagttg acattttaca aatctaaatg acatctttta 44940
cattctactg acccccaagt ttgaatgcga ggaacccctc attttaagct tattataaat 45000
gggtattatt tggaaaagat ttagcttcta ttcagttact ttattaccat ttaacactta 45060
tagtactgca tttatttatt taatcacata tttgtttcct tattatttaa ttttctgtat 45120
tattattgtt aattttatct atatgttaat aatatttagc acatttaact tgatgtgtta 45180
atgaattagt atattaatga tttattattt atatataaaa tctacagata taaataatag 45240
atataactac tacaacgtat taatataagg gtaacactac aataagggtt gtattttcat 45300
tattgttagc taatccaatt actaacataa aaaattacaa aacactaagc atcacagtat 45360
tttttgtgtt agttaatgtt aaagaaaata catttgttta ttgtgagttt atgttactct 45420
tgcagtgcat taattaatgt taacaagcat gaatttagat tttaataatg cattaataaa 45480
tgctgaacta tggttaataa ataccgtcca agtatactta gttaatttta gcaaatacat 45540
taacgaataa atgcttactg taaagtctga ccataattat tatatatatt gtaatcaact 45600
ttaggattat taattttatt catcttaata aaaggctagg taatttagta gaaacgtgtg 45660
gttttatttg aataattaaa tgaagaattc tttttaaaca tggacccccct ggaaaaccct 45720
tttgacaccc tgagggttca caaaactgag atggacatct taggcagcag tattttactt 45780
gtttactagt aaatgcatta gctgagatga tgtagatgcc aggagcaaag catgtcttac 45840
ctcatgctcc tctccctcat gattgatgct ttctgttgag ttctttttgc ccagaactga 45900
aacacaacaa agagaaacaa gaaaagtcat tgctcaatgc cctgcagaca ttattcattt 45960
atacacaatt attcgcaagc cgctaaacca aactgggcac tttgataaag cacaaaacaa 46020
caaggaattg aaatgtatct gaccagcact gtaactaaag ccacaagctt gttcccgtca 46080
ttaattccaa caaacaagct taatctagtg agaagactca cgcctgtggg ctgctcaatg 46140
catcactctg tttgagtgcg agcacaccta attactaaga ggaaacatca gcttaaccca 46200
tccagtcatt tcaaatccct caagatgaaa atcagtggcg tcaatgagcc actatagatc 46260
ttactgaatc aatttagcca cttaatgaat gcaataactc agatgagcat tgtttaacac 46320
ctctgcatta caagcttctt ttttagtcaa attgctgaca ataaaacaaa gtctgattga 46380
ctttgtatgt ttgcaaactg atctgttggt aaatgtttat cttaaaaagg ttgcaatcag 46440
aatatgcaca ttttacaatt taggttcagg aaagattgaa cttaagttgc aaaatatgaa 46500
tactgaactg tttaaattac taaacaatct aaaagttgat caaataaata cattgtttat 46560
tttatcagat atacgaaaaa tctgaacaag gtacaagagg ctgattattc attgacccga 46620
ggcagaatag ttagaactgt ttgaaagata aaataaatct caaaaaggca ttctttcaaa 46680
aaaatgatta gtctgcaatt caaattcaag taagaaagaa aaatctgata gacccgatat 46740
agaatacata tatgacaaag gcaaatatag atgagagcct gtcgtacctt gatctgggtg 46800
ctctggatga gtcgaccctg gatcgattct tccgatcatt gggtcctaga agaatagtaa 46860
gaaggtcaag gacagataga ctgagattca gagtattatt agcagtaata gcatctaatg 46920
taaaggtctt gaactagaaa aggaatgtga attgtgaata ggctctcttt agaaagtgct 46980
taatccaaca tgaattatgg attactttta gctttctttt tttaaagtaa taatgtaaat 47040
aatttcagca aaaaatcagc tttaatgaag aaaaaaaagt cacatacata aaatcaaata 47100
tcaatataca taaatcaaat gaacaacaaa ttttaatttt agatgaacaa tttaagtaaa 47160
atgtgaaaac aaaagtgtga aatttgtgaa attgcattta aatctctcgg atgttttgtt 47220
gcaatcctga gctttataac caaccacatt ggttggtttc acttacatgt ttgtttgcca 47280
tttcagattt tttttaaatg catttttttaa agagtcattg agatatacat attactgcta 47340
aaaatactac aggtaaaata aaaccattat ttatgtccgt gcacaactaa aaaataaaca 47400
aatttaagtc catgaatgaa gaaagggcaa tttatagatt ttttttctta tcgcgattac 47460
actactcata aatgttgttt gaatttgcca catacacgca ttgatcgaaa catccatttt 47520
attgtttact gagttcttat tcattttgct gtaaaaaata aacacactaa aaaggaatta 47580
tgattgtatt tttaatagga aactttggat ttctagtgat ggttattatt ttttaaactg 47640
caaaatataa ttgaagctag acacctggag agccacatat ttttggtcaa atagccatat 47700
gtggctagcg agccataggt tccctaccac tgttgaaaac agtttgttaa tcacttttta 47760
ttgttcattt ttaattgcta atagttttaa ccatagaaac cttaaattgt aacttcattg 47820
acactaatta cattattatt tttattttaa gccttaaaag ttaccctagt aatattccac 47880
tcatttttttc aatgagcatt acaatgagtg gcctataaag ggttaattaa ttgaaggatt 47940
tgacacaatt gataagatca tgataataaa agctcaatgg aaatcaacag agaaggcaaa 48000
tggtgcaaac tattagaaaa gcaaacttct gaccatgcga atgcctcacc tgtattacct 48060

gtctaccaca ttctactgtg accagcttat ggcatttctt atgcaccaga agcttacagt 48120
tgatacactt gtatccctgc ctgcccagac cccagatacg atctgtgcag atggcacaat 48180
gagcacgctg tacaaaacaa agtgacaggc aaattaaagc aaacacaaca tactacagag 48240
aaataaaaaa ataaaaataa aaaaaataaa tatatatata tatatatata tatatatata 48300
tatatatata tatatatata tatatatata tatataataa gagcatatac cctgttaaag 48360
cgtttggcct gaaacgcatg tccagtggca tagtagagtt tcctccaacg tcgagctccc 48420
cgccggtata tagactctga gtacaacaag tcaataaaat ctatgcacca cttacacaga 48480
atcaatgcag ccacaaaatg tatttttttt ttaaatatga tataaatatc agaaaacaca 48540
catactgtct tctccaggac agggcatgcc aggtttttca gggacacaag gaaacactgt 48600
ggagagagag agagattgtg aaaaaacagc tcctgcatga cccctctgtg acctctcctc 48660
gtctgacagc ggataaggac tttgggtcaa caaacagagt ctgtccaggc ctacaaggtc 48720
agccggtgtg gacagtgtct attgacggac agtcggtggg ggtgtttttc tgaaagccct 48780
tgtggccagc cgtcgtctcc tcagcataac taatgaactc acccagcagg cagaagaaca 48840
atgcatctct gtcaattagg cttgcatgat acttgtgata ttgtaaaatg tgatattgtt 48900
gagttttgcg ataacaatat ttcctgtgat ataacattta ccaagagaaa ttttatttta 48960
ttagctatta tttaaaatca tttatgtaat gatcctagta catatctatt tgtaattcct 49020
gtttaaagca aataactatc tgtatattct gttcatatct atctgcatgt accgaattat 49080
taatgaaaac ctgttcagta tgttaatcta taagtaaatc tcttattata gttaagaaaa 49140
cttatatatt atgttcacag tggatccatc tgtaaaaatt acccatagtt ttctatagtt 49200
gcactcataa tgtctaccta tatcctggac tcctggatgg acctaaaccg catttcgttg 49260
ccttgtactt gtgtaatgac aataaagttg aatataatct aatcaaatct acgaatataa 49320
acaggtataa ttaacctagt ttagggggaa aaaacaagct atgaacattt agactttaaa 49380
acaatgtgaa gactataaac cttaacatga cattttttc ttattgccat ttccctgctt 49440
ctgatattag tatttatttt tcaaatctct gctctgaggc actagattca cctttggtaa 49500
taatagcatc tactgggtac aaaaataatt acaggtataa aaaagataaa ccctatataa 49560
ataacctttg catgcaacac aaatgcttgg aacataaaat aaagtgaatt taagtcgcac 49620
ctctgtgcaa tacagatatt gcacatacga ttatcgcaat aacaataatt tttcagtata 49680
agcagcccta ctgtcaaaca ttacccgaat actttctcat ttgtccaata agcatctgaa 49740
gacctgtatg actctgtagg gcttgttatg aataaataaa caaacacata tacccgagcc 49800
ataacatcaa aatgtggagc ttcatcagag ctcatcacta gggtcttgac atgcttgaaa 49860
gaatgcgtgt tattatgact catgtaaaaa taaactgttg atggaaacat taagatgtgc 49920
ataaatggaa atcataaact gagtaaaaaa tgcacaaaaa ctatgatgga aacactttta 49980
ccaaacaaat tccactatac gaataaaaaa aaaaaagtca tgtgattttc ttttaagaga 50040
tcatgtgatg gtaaaaatgt gtgtgaatgg ataaaccagc aggttaagca cattgtaaaa 50100
catctgaaat gttgttttgg tcattctaaa acgccttaac catttcagca tccacgtgct 50160
ccatgtctca cgccttcaaa cggcaccaca tgttcactgc aagttgggat tgccttctga 50220
ggtgcaagtc atttattaaa taaagaaaac aattacgcaa aaaattccat ttttactgtt 50280
gatatttggc agcagttaat cagacagtga tgattttgtt ctctttgact ctgcgttatt 50340
tgcatgtact ttatgcaata ttccagtttc gcgcatacat ttaatttgca tctttggatg 50400
taaacatagc taatgacagc aaaagttgcc aattaaaatg cgaagaacac aaataattct 50460
aaagccaaaa ggtcaactag aaaaccttct cattaagatt gtgttacttt tttcacattg 50520
ttcaggttaa ggtgcataca aacaatcaga aaaactatat gcttgttact atatttgata 50580
gtgcattgaa ataatctcag ctttcaaatt atgataataa tgaactataa taaagtttat 50640
aaactggtaa aagtacagaa aacaccatta acaataactc caattggttt atttcaatgg 50700
aacctttca taaacctgtt atgaggcatt aaacagttgg gacaggacag caagtttact 50760
gttattctca cacaattgtt ttttcctttt tctgaaagtt tttataccat catcgtggag 50820
tttttctttt attatttgag caaataagat ttttttcca aactttttt gtattctcct 50880
attctctgcg ctctaagttt ttccaattat gatgacttct gctactgaga aacctggaaa 50940
tgtgaaaagg gtctattgtt tctctctttg atttggtctc tggttaattt cttctgttca 51000
gtctgtcatg atcagacatc cacacagagt tgcttaacag cagaaactga agagtagctc 51060
cacttaaaaa caacaatgat tgtttatcat gtttgttaca gtaaaacagt ttgatttgat 51120
aaaatctact ctattaagtg ctatttacta gggctgcaca acattggaaa aatataacaa 51180
aattttgtta ttctccaatt atatattgca atatgaatac aatttctcca gatgacttta 51240
tcattttagt taattgtttg ggataattct gcaggtgcat ttgcaaaaaa cctaagaaag 51300
atctctacaa gcataaaaaa atgcaataaa aaaaattata taaactgtta ttcattgttt 51360
tcctgaatac taacagtaga cataataata ctgcatagtc tttgttgtat aaataataca 51420
ataaaattat tggtatttaa tttaaaattg gttcaaattc ttatgcctga atgtttttaa 51480
atccctttaa cggtcacatg cctcaaatat gaatgcaaat tatgaattat aatccaaaat 51540

caacattgca tatcatgcat tgttattgta tattgtacag ccctactata tacaacaaac 51600
aaaggtaatg taactgatgt atcaaattac atctatgtct attttcttat tgcgaagaaa 51660
accaaactac atatttacat tctgatctaa acagtgtagg atgatgacgg gatgttctct 51720
gctcagttta ctgctgctct gggcatttaa atttttttcc agaacaaaaa aagaactttg 51780
ctgcgagtac atcggggcct taagacctgg tgtaaaactt catccctcat aattctttca 51840
agaatcgaga gagcacaaaa gcaaacgcac tctagctaac ccacatcaaa agccaaatcc 51900
cagcataccg tgaataatga gctccgagtc tttgttgagt tcatatagac gcaaggcctc 51960
ctccagctcc agctgagaag aaacggtgca cggatcccct gaaatgacaa gcacagagaa 52020
cgctcaaaca caactctcca ccaaagacat cagcataact gacattgcga agatacaaac 52080
gaggcgagtg agggagaatt taagcaggcc tgagaaagag aaaagaaacc gagcgctctg 52140
atcatatgga gcgtaaagaa aatatctgtt cgattgacat cataaagccg taacacggcc 52200
catggtgttg tacgaagctg acgtcaatgc tgttgtctga agagtagcct gtggcaccaa 52260
cttcttgcac agacgcacac ggctttcaat caaacatcac agagaaggca atgagcgacg 52320
gcgggcatcg ctgccaaggc ttgcgcagac agacgggatt aacttctcgc catgacggcc 52380
cgctcagatc agctatgcta atgagcagcg tgactctggg ctcagcgtgg cacggtgaag 52440
atttggtat ttaaatgaat tttcttccat ctccatttcc catctcgcat gactaatgca 52500
gcagttaatc atcgcgcgca gtcaatcttt ccagcataaa accccgctcc ctgctgtgtg 52560
ttgcattcag attcgcatta ttcctcatgt aaaagccatc tattttagtt ctgccggtgg 52620
agtccagggt aattcggaga ctttaatgca atctgccaaa agtggcttca tttgtaatat 52680
ataaagtgct ggtgcaaatg aggcaggcgc ttcaacagaa tggcaggagg aagcttgttg 52740
ctacggcgac agttaaagaa acagaaggaa aggccacgtt tacacctggc attaacatct 52800
gtctcaaggg atttgatcaa gcggacagca ctaaatgcag gttcaaataa agtgcaaaag 52860
attttgtgat gactcgaatc ccatttgtgg ttagttaaac acacacactt tatttatatc 52920
atcaatgctg atgcatccag gacatacaac acaaaattac atattgcata agatgcaata 52980
acatattaaa atatgataat aacaacaatt tagtatgttt aatgtctaaa ttgaatgaac 53040
tgtcttgaaa gaattcagac atgatctact gggattttca cacacaacca tttctagggt 53100
ttatagagaa gggtctgaaa aagaaaaaac atctagtgag cagctgtttt gtgggtgcaa 53160
atgccttgtt gatgccagag gagaatggcc agactggttt aagctgatag aaaggcaaca 53220
gaactcaaat gaccagtcat tataactgag gtatgcagaa gaacatctct gaacgcacaa 53280
cacgtcaaat ctgaggcaga tgggctacag cagcagaaga ctacactgag tgccactcct 53340
attagctacg aacaggaaac tgaggctaaa atttgcacag gctcatcaat atagtttgga 53400
aaaatgttgc ctgctctgag ttttgatttc tgctgcaaca ttcagatggt agggtcagaa 53460
tttggtgtca acagcatgaa agcatgtatc catcctgcct tgtatcaacg attcaggctg 53520
gtggtggggg tgtaatgtgt gggggatatt ttcttggcac actttgggcc cattagtacc 53580
aatcgagcat cgtatgaaca ccacagccta tctgagtatt gctatgttta atacctaaat 53640
tgaatcaact aggagcaagc aaaatcatgt cttgaattaa ttctggcact aatatggccg 53700
ttcagcatta gtataacatc gaaaagtgca ttttattgtt aaacagtaaa ataaacatta 53760
aactattcat gaaggtgacc cttattctca agcaactaag acgatagcct ataaaacagc 53820
tcatttagaa aaatgggtca attaagtgta atagccaaaa ttcagtttta acaagtaaaa 53880
gctctctttg gtttaacaag ctgtgtgtaa tcatgaaaag agagagaggg tctctttagt 53940
gaaacctgat ctaaagaagt cactagagat tcatgttaac caggagtaaa tgtcaatctg 54000
tctcgactga ctgagatcac ctgagacaga tgccaacacc tactgtgaat agagcaaaga 54060
gacacaaaaa tgaaacaaca aaccttcctc atcaatccat ttcatggtga agagctggtc 54120
attgtccatg gagcacatat cacgcacctc attgcagagt ccctcatagg agatcgaagg 54180
ctcaaaatgt gtgatcatga tgtccctaaa acagaaaaaa aagtgacatt cattaaaatc 54240
ctacatttta gggctgcagg atattagaaa aatctgacaa tgcaatgttt tgtttttctg 54300
tgatgtgtat ataatttccc cagatgactt taaatacctc gatttggtat gaaaccatat 54360
acagaattta atcaaatgta aaatagcagt gcttagattt cattgcataa ataaatcaat 54420
tgaatatttt ttattaaaga ctggtacaat tttcatgcct gaatgcttta aactctcttg 54480
atatgcttta atcacaggcc tgaaaaacac atgcaaataa aaaacttttg ctttattatg 54540
gaataaatct tcaataactg tggctcatgg taaactatat tcacaaattc aacattgcag 54600
atcctgcaat ctgactacta cagatgcaca tatttcaata tcaatgttaa aacaatatat 54660
tgtgcagccc tactcttgaa aaattaaggt ggggcgagag gaaaaattta tttaatcaat 54720
tcatagttct ctaatatata ttagggatgc tctgatcaat cggctggaga tcagatttga 54780
tcggtcctca cttatctggc tgatcacatg aaccaaactt aatgttagat tacaggttta 54840
caagcagagt aatttacatt ataacttatt atgcataaaa aatgcacttc gaactttttc 54900
ttcattgagg catgttgact tattcttcca tcatttgttt ccaaattgca ttgtcagtca 54960
tggtacccaa ccccacccct aaacacaact gtcattgggg gatgagtaaa ttgtacttaa 55020

ctgtaccagt gagattgtaa gaattcccac cccatccact aaatcaaaaa cttctgaatt 55080
gtctagagat agcattggtt attcatgttt ctatccgcat ccctgtaaca gcatgcaata 55140
ttgtgcttat ataaagtatg aaaaagtacg actgtataca atataattta ttaatccaaa 55200
aataaacaat aaagggtaac atttttattt ttatgaccat tcgatatata tctataccat 55260
cataatgcac agccctccta aaaatatgat aaataattta gtgtgggata ataagagaaa 55320
tctaaatact atattagtac attttaagac caaaaacaca gtacatggag tatcagagat 55380
ggcgagtttc aaggaagctg taatactagc atattcccat agcaggaggc acacagcagc 55440
ttcttactct ctgttgtcta ccgagttcac actgttttgg ttaattatta acagcaggga 55500
gtctttcctg tctacgagcc agtgcactga cccacacatg cctgcgagcg gccactgcaa 55560
ccatcccaca catcacctta tgacgaacca aaaaaaaaag tgctgctttt atctgatcca 55620
ggactattac gatcaagcta ctgtacgaga cataaaaggc agttatgcat gtcctgtgac 55680
aaatatctct gagattataa tagctccgca ttactaaact actagtgttt agttcatatt 55740
tacagagaga gacagattta ctcttatcat tgcataaacg atataacaat attctggcat 55800
ataactgaag gttttgtgcc cactatctta gtagtgagag atgcatcctg tgaaagcgca 55860
gcatctctga aaggaagcag caaagccaca ttcctctgta gggcatacct ggatcagtac 55920
acactgcttt tacaacaaca agcatgatga gcaaacattc agtcccatgg tcaaatacga 55980
cttacaagaa actcaaacac gattttctga acctgaattt gtaaggaagg cttacgggga 56040
ctgaagtcaa ggcactatac taggaatagc acacaaggaa atatgaccat tcctttaaag 56100
caaacaatag gtgtattaaa gggaaagttc gctgaaatga tgtcatcatt taaggtgctt 56160
tcatactagt ttagctcata ctttgtttta acttcaaaca atttatagtt ctgatgagct 56220
ttaagaatgt caggtgacac tacgttaagc aaacaatgat cattaaatga caccacactt 56280
tttaaaaata actataacac attcaaaata tgaccttaaa ctttccagca tcttttaaaa 56340
aaaaacacaa ttcaatatat aacttccaaa atggtaaata tgcattctgt ggcacttttt 56400
accaatgcta acattgcagc atttcgact ctggtattaa actatacctg gcaacatcat 56460
gactacaatt aaaaaaacaa gtgtttgctg cacattaaga ctgccagatt ttacattcat 56520
ttaagttata ctgcaccaac agcagtggtg gttcagatga cataatagtt tttgagtcac 56580
ggatcggacc atttttcgga tcagcaaaaa aaggaaggag acaaatgtca attgctttca 56640
attcatgcaa aaatataagt gcaaaagcca ctggttttaa aaaacagaac ttagaacctt 56700
taattttcat aaaaatctca atttaaaaac aaccggctga aaaaaaaagg aaaattttga 56760
atacgaaaaa tgatctttgc tactttagct gatgatgcta aatatataaa tctaacatat 56820
tgtacagaaa atcatcttta ttttcaatta atgattcaac aattcacact taaagtttca 56880
tgtttcatta aagttttatc atttttgaaa accggatttc agtctttacc ataaacatca 56940
gtgtctttat ctgaactata aactcttctc ctgtgttatt taattgcttg taatgaactg 57000
aaagtgtaaa aactgaatct ctctcggtca aacacagatc tgaatgcagc actttacact 57060
gaatgcatta attcaggcta aacagtgaca gaaaacactt ttaattaata acctaagaaa 57120
gcatttacgt aggtcccacc acaaccgtaa tccataatca ttatatttca caaggaagcc 57180
atgatgcttt catacatgtg atttgaatga cactcgaggc aatctgtctg caattgttat 57240
aaatgttgga ttaacctaca agtttaaaaa gttaataatc cgtcattaaa catgtgcgtt 57300
ccgaactgtg ggttgtgatc tgtatggatc acgaacaaac cgtgatcggt tacacccccta 57360
accaacaggg ggacaataaa tattttttta ttttaatttc aaattaaaag gagctgtcaa 57420
aaggtggaaa aaaaacatat tgaccaatca atacagattt tttacatttt ttaaatgtcc 57480
aacccatctc attgttagac atgnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 57540
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 57600
atctctgttt tttattatat atatcctggg gtatcccttc aagcaagaaa aacaggggac 57660
taaaatgtag atctatctgc agaattttca ttatctcaat ctttacccct tgccaaaatg 57720
cacgtatctt cggacattcc caaaatatat gtgcgtgatt ataatgagct gtttttaatg 57780
tctaaaaatg aatggaggta aataagacct gaagtctcga gtcaaaaaga ttcaaatggc 57840
tgcaaccgct tgttcatgaa gaataaggta aataaatgca ctcatgcaac atggattttt 57900
tgctttaaat gctattacag tatttaatgt taattacatg aacaaataaa actttacaat 57960
taaagtgtta ctataaacat attaaaattg gaattattaa aattaatatt aacaatttag 58020
tgttctcaca aattagagat tggtaatgca tataaaaaaa tcaagaaaaa gtttaaagaa 58080
aataatttta agtgacaatc taaactattg cagacagaga aggggccacg agccaagcac 58140
atctctactg tggaaaatgt tttagcttaa tagttgcaat ataggtcaaa tctgaaatgc 58200
ttattacaga catactacat gtgaccgaga gaacagaaaa ggatcatgca tgagtgatag 58260
aaagagcaat aaaaggaaga actaaggcaa gaggaggaga ataaagactg aaattcatct 58320
tagccaggct tcaggggctt tttaatcaga atataaaagg tgcagagagt aaaatgtcct 58380
tcaatttatt cagtggtcta cctgcagtgg ttctcaaata gtggtatgca taccaggctt 58440
ccttcgagtg gcacacagac tatcgctgac taattaaagt aaaaaaatga acacaccttt 58500

caaccctttg atgcatatga taacacctga tgtgattagt aaattgatag gctaaacctt 58560
ttattttaat ttttatttta ttatgtttgt tatgtattct atcatctgaa gctaatcttc 58620
tcccaatatt tgtaacgctt gttgggggcg tatccgtttt tttaatatcc cattgttgat 58680
tggtatgatt taatgaactg cttttctgac acacaaagct tactctaaca cgcagtcagc 58740
agatctaatt gaaaatttaa atatgcaaat catctatttc aaatatgttt agatttcaaa 58800
gataaacaaa tgcatgcaac atatttcaaa aatgaaagat tttgaaaaaa agagtttata 58860
tacgaatatg atgacatata gcctatattt atgaggccca aggaacattt cctggtttta 58920
atgaataggc ttttatatgt taatacttta catttaagta cagcagtttt cttttaactt 58980
tttaagaaca gtgctgtttt aatctacttt taaaagcaca tttaactcaa acattattta 59040
ttttttttaca tgtgagcaca gtgcagtgta aatgttccaa cgatttaaaa tgttttaagt 59100
ggctgacaat aataaatact cataatagaa attaatctgc ccgcttttga actgtgcaga 59160
gttgtagctg cttaattagg cctgctacgc tactgtattt taatactgat cataatggtg 59220
gtacttggag atagggctat gcgattaatc caaattgaat cgcaatcaca atttgaaaag 59280
ttgtgattag ttaaatcgca caaggctgca atataaaata tatatgtata tatgtaaaca 59340
aaaataataa ataacatttt caaaaacagt ctgctatgct ttagaaaatt acacatgcta 59400
gacattctgt gatagtgttg tgaggtatcg cagacatggt atcatttttc atgaactacg 59460
ccacattaca caagaagaaa agactgaatc ttgtatcacg aagtccagtt gtcctctatg 59520
gcttcccatg acaccctatg tcaaggaaat cgtgccgaaa tcatgtgatc tgaccggggc 59580
tttataacta gccaactgag tgagcaattt cattcagctc aatcagaaat gcgcaactga 59640
acttcggcca caataaaaaa agaaaacaaa caggaaagcg ctaacaagtg gaattatagt 59700
gtctgcttct ccagaagcat taatagacaa attaatataa atgaaaaaca gtatttgtta 59760
tatgggaata ttttggtttc aaagtcacag acgccaaaca aaaataggca atttttaaga 59820
gctgttgcag aaatgtttac actgcttaaa gactattaag ctaaaactaa aattaaatta 59880
aaatcaactt gaagcttgta ttaaatttta aatcaagtcg caatatgtca aaaaataaat 59940
aaaaaaataa atagcaacta gatatattgc ccaactggca cagccctact tggagagaca 60000
attttttctg aggtgatact tgctgaaaaa agtttgagaa ccactgcatt caggcagaaa 60060
cctgccatta aggcctccac acaatccctg attccctcaa tcacatccac agtagaagtt 60120
tatatggaaa gggggttgaa actgattcaa atactgtcta aaatgaccta ctggaaaaac 60180
aacagaaacc agagaaaaca cacattcaga cacaaatggt gggtttcaag atagtccaca 60240
ccctgaaatt ctgaccgtct atttttatac ggcatagacc atcaattcat gctcactcca 60300
gagttttcaa aaggatttca tatcctaaag gatttaactt tattcactga aacacagatt 60360
tccaaactaa aagattgtac agtggcctgt ggcatggtaa acctgtcggt tccttacaat 60420
gatggtaaat aaattcttat caacaaattc acctgaaaca gaaactgact agtgtcaaga 60480
caaatgcaca tatactatag aactgaagag taaaaagagc aatcagtctc caacaggtct 60540
gacaaaagat acttgtcata caagtttttc aggtgcatga caagaggaag cgtcatcacg 60600
aagaaatagc tgactaaaaa tcagttatac ataaatgtgc tttattattt acatcaagta 60660
aaaatatttc ataataacta caccttttc caacaggtca cttttccccc tagcttacac 60720
tgttatacag aatcttctaa atgtgacaga tagtgagtcg gctttgtgac agggaaagcc 60780
ctaagatgaa acaacacacc atataaacca ctgttatctt aacagtaaat cactgtgttt 60840
atgtgaacaa ctataggtga agacgaaaga cgagatacag agagagtaat gaagaaacag 60900
ctcatttgtt atggttggtt tcagttgaca acttcatcag agtttgtttt tgagcttagc 60960
taacgtagct tgctagctgg cttttcattg gtttctgaaa atttgcaccg ttgagaataa 61020
gtgagaatgc aattgtatat ttaaacggtc tcactggcta cttaccctct gtagtaggct 61080
tttacccgga cttggtgcgg gttttctccg gggtgggaca tggtgctgtc ccgcagcgtg 61140
ggcattatgg actatccgta ctcctgctag cttagctaat gatgctagcc agctatcgtc 61200
tgacgaggaa aaactaccgg agttttaaaa ctaaacgtat tctttggaca gcaatagtgc 61260
cgtctccggt taaataaaca gcctggctga aatataatta gtgaagttca gacggaaatt 61320
aatcggacta ctagaagttc tgtcagacgg ggaaacactc ccttcttctg tcgcgcaggc 61380
ctcgagacgg actgatccaa acagcggaat gtggaggggg aggatgagag ggggatttcc 61440
agaagaaaaa tattttacag taacagcgat tgattgtcac acaacaacaa caaaaaaaac 61500
ttgtgactgt agttttaaaa agggtacaaa tgctaggcat ctgcaatatt gctgatgata 61560
gagtattatg gcaatatggt tcaatgggtc tagttgaatg ggagagtctg gtaacattcc 61620
gattatacca cacagaaatt gaagagggtg gagaacagta gcctttatag gaagtacatt 61680
acactgcttt ttgtgggtct atgaataatt gaatttggag ggacgcgatt aatgaagaaa 61740
ttaacgcttc tgtttaattc ttaccttcta cttcccagag tgaacgtact aaaaaatcca 61800
gccgcaaaaa cttcatagcc ttttaatact cggacaaggg cagtttctta aactgtgtac 61860
aatattaaca ggggcagttc ttaataatag agggcccata tacctagata aataaaaatt 61920
gtccctgttt ttctttatta ggatgttttt acttttgaac ttgtaaatcg tcacatgttt 61980

ccacatcaga aaaaagcctt atatttaacc aataaagcaa aatcttagca atgttaaatg 62040
acatctcctt taaccaataa aacttcaaaa tatttgccat ttgctttata tagcctaatt 62100
ggaaaacaga aactaaatga gacatatcag cacacattat gctctcaagg cacgatggta 62160
tgaaggacga aacctgcaaa aacaataaat aaatacgcca atatataaat aaattagtaa 62220
ataaatctat aagtccctgc ataaataaaa taaataagta ataaataaat aatcaaataa 62280
tcaaataaat gaatattaaa atccacaaat tcctgcataa ataaatattt aaatgaataa 62340
tgcgggtagg caaattatta aataaattac acaaaagttg aggaattagg aaaatgctaa 62400
actgaaagtt tatttaccct ctttcaaatg tatattcatc tattttcatt agcatgttga 62460
cttgaggaaa agtaaataca gaactatgtt caagaaatga ataagcgttt gaagggggaa 62520
ataatctttt acttttttccc ccaatatttg tgtaatttta tatacattta tttatttgca 62580
tatttttttt ttataattta ttaacacaat aatttctata gggaaaacac tatctattaa 62640
gctaacatct attaagtctc ttcttcatta agtctcttct gctcaccaaa cctgcattga 62700
ctccaaagta caacaaaaac agtcctatta ttattattat tattattatt attattatta 62760
ttattattaa tattattatt attattatgt ttatcaatgt ttattaacat ttaaattctt 62820
tttttgtttg atgtttaaaa atatcgaaag accagcattt atctgagata aaaatctttt 62880
gtaacatcat attaaaacat tgaatagctg aaagatagtt tttttgtcaa agattattgt 62940
aaaattaata gttttatata ccaaggatac tatgaacaaa agtgaggata aagtcattta 63000
taattttgta catttttaat tctggcaaat gcagttcttt tgaaatttct agtcattaaa 63060
gaaagctgaa aaaactcctc cctgtaagtc tgtccgcttt gcccaccagg atgtagatgt 63120
gtgtctgatg gactgaaatc ccacctgaaa gaagcagagg aacaggatgc taaaatactt 63180
agaaatgcaa atgaatatgg tattagcaag ctatcaaaaa ataagcagct gtgatcattg 63240
taatgtgttt tgtctctgct gtgcagtctg ttgtccagcg gagggttccg gaaactgtgg 63300
gatggcttct gaaggttgtc tgtcgtttca agatggccaa ccattctggc tcaagctcaa 63360
gcttctcgaa ggagcttcct ccacaatctg caataacaaa acaactgaat ctacttctgg 63420
atctcacttc atactaaaac atgctggatg atggaaacag caggatattc attattgtta 63480
gcctgaaggg acaagcagct tgttattcat tttaaactca tatctatatt ttattcttag 63540
cctttttcac acatttcatt tctgaagcct gtaactgcaa gagatataaa agatttcaca 63600
tcgtcataaa caaacatgca agtcttttac aagtaaatta taattttttta tttaaaataa 63660
cttcaatgac actaagtttc aaatgtggat caaaaaatat tccgtgggca tatacgttat 63720
aacagatata ccgtacactc actggccact agtccaactg ctcgttaagg caaatattta 63780
atcagccaat caaatggcaa caactcaatc catttaggca tgtagacatg gtgaagacga 63840
tctgctgcag tccacaccga gcatcagaat gggaaagaaa ggtgatttaa gtgactttaa 63900
atgtggcaaa gttgtgccag acggcctggt ctgagtattt cagaaactgc tgatctactg 63960
ggatttacag agggttcaca aagaacgatc agaaaaagag aaatatccag agagcagcag 64020
ttctgtgggc gcaaatgcct tgttgatgcc aaaggacaga ggagaatggc cagactggtt 64080
ccagctgata gaaaggcaac agtaactcaa ataacccctc gtttacaact gaggtatgca 64140
ggagagcatc tctgaacaca caacacgtct aacctagagc tggataggct acagcagcag 64200
aagaccacac cgggtgtcac tcctgtcacc taagagcagg aaactattcg cacaggccca 64260
ccaaaattgg acaatagaag attgggaaaa tgttgcctgg tctcatgaat ctcgatttct 64320
gctgaggcat tcggatggta gggtcagatt ttgctctcaa caagatgaaa gcaaggatcc 64380
atcctgcctt gaatcaacgg tttaggctgg tggtggtgta atggtgtggg ggataatttc 64440
ttggcacact ttaaacccat tagtaccaat tgagcatcat gtctatgcca cagcctacct 64500
gaatactgtt gctgaccatg tccatccctt tatgaccaca gtgtactaat gttctcccaa 64560
cttccggcag gaaaaagcgg catgtcattc atttattctt tttcttttcg gctaagtccc 64620
tttattaatc catggtcgcc acagtggaat gaaccgccaa cttatccagc ataagtttta 64680
tgcagcggat gcccttccag tggcaaccca tctcagggaa acattcacac actcattcac 64740
actcatacac tacagacaat tcagcctatc caattcaccc gtacctcatg tctttggact 64800
gtgagggaaa ccggagcacc cggtggaaac ccacacaaac agcctacaac agccgacaaa 64860
tcatttgcaa ctttgcaaca tttgcagccg acaaatctgc agcaactgct tttttgatgc 64920
tttcgtgtca atatggacca aaatctctga ggaatatttc cagtaccttg taaatgtatg 64980
ccacgaagaa ttaaggcagt tttgaaagca aaagcgcgtc caacccggta ctagtaaagt 65040
gtacagtgag ttttacaaaa taatatgggc ataactttgc catgatgaca acacataata 65100
ttttactaca tattttcaag atactattca gcttaaagtg ccaattaaag gcttaacaag 65160
gtaaattagg ttaacagggc atgttaaggt aattaggcaa gtcattgtat gactgtggtt 65220
tggtctgtag acaattgaaa aaaaacatag ctttagaaaa ctaataatat tgatcttttt 65280
ttttacccag aagaaacaaa taaagtaaaa taaaagtaaa taaagtaaaa tagtgtaaaa 65340
aaaaatcctt cttctaataa acatcatttg ggacaaattt gaaaaagaaa agaaatttac 65400
aggagggcta atcattttta cttcatatta caatgaatgc catgtagtgt gtgaaacagg 65460

ctcaatccaa gctaaccaaa agctttcgtt tgtcaaaagc acagagtgac agtgaatgag 65520
gactcacctg gtgctgcatc agagttgagt tgagtgcagg tgagcagaga ttcagtatct 65580
ggacttcagg tgttctagaa gctcagcagc agcagcagca gcagggcttc ccaaagtact 65640
gctctccact tcctgtctga ggaacttctt cctgtgcaca agagcatttg tgttgctgta 65700
tactgataaa tggctcacga ataatcatgc agcaggaaga tgactttggg catctacatc 65760
tggagaaatt cagtgagaat gttttaattt cattagagag gagtctgatg tgcatcaaat 65820
ctattctaat ccagttcctg ggcgaggcag tggcgcagta ggtagtgctg tcgcctcaca 65880
gcaagaagat cactgggtcg ctggttagaa cctcggctca gttggtgttt ctgtgtttct 65940
ccctgccttt gtgtgggttt tcttccaggt gctctggttt cccccacagt ccaaagacat 66000
gtggtacagg tgaattgggt aggctaaatt gtccatagtg gatgagtgcg tgtgtgtgtg 66060
tgaatgtgtg tgtgaatgtt tcccagagat gggttgcggc tggaagggca tctgctgcct 66120
aaaaacttgc tgaataagtt ggcggttcat tccgctgtgg cgacccccgg attaataaag 66180
ggactaagct gacaagaaaa tgaatgaatg aataatccag ttccagctgc ttttaacaaa 66240
acagtaaatc tgactgcttg acactgaaaa ctggatttat aaaatgcatt gcaataatta 66300
taaaacacta gatgtcattc actttaaaca aactaggtca aatttaaagt cccccctctat 66360
tgcacaataa tggaactgtc caactgttgc tgtccaactt attattatta ttttttttaa 66420
atgggcttaa tttaaattta ttaatttaaa tgggcctcat tgcaaagtaa atgattttag 66480
attctctgct tttttttgca atttatttat ttatttattt aaaaatgagg attggatagt 66540
aaaaattgtt acacgttaaa tcaattactt tcacagagta taaatataag aaactagtta 66600
ttatacactt agtttaagag taggcactaa ggcccaaccc tatttcacca aggtccactt 66660
attttaaaat gtctggcctc tcccctcact aatagtagtt atatagtatt aatctctagg 66720
tcaggtggtt ctcaaactgt ggtaacgcgg gcttcctgat agtggtacgc ggagatatca 66780
aatgtgtcat atgtacatgc tacatatatt tcaaaaatga tttatatatg aatatgacat 66840
atcatttatt ttattacata ttgcctatat ttctgaggtc caggcaacat ttccaggtgt 66900
tgatgaattg gctactatat gttaatactt tacatttaag tacaacactt ttcttactac 66960
tttttaaaaa cattaaccta acatttttaa cttttaaaag cacattttaa tttaaatgtt 67020
gatgttctta gtttttatgt ttgcttgttt tttttgtttt ttttacatat aaacacagta 67080
cagtgttaac attcagacaa ttcataatgt ttaaagtggc tgacaataat aaatattccc 67140
aataatagaa attaatctga tacgttttag aactgtgcag agctgtagct gctttactgg 67200
gcttactacg ctactgtatt tcagtactgc tcattatggt ggtacttgga gagacaattt 67260
ttttctgagg tgttacgtta tgaaaaaagt ttgagaacca ctgctctagg tcaataatat 67320
ttatttaatt aaaactatga actcagagct ttatcttatg ctcatggtta cgagattaca 67380
gaaattcacg taaattaggt caaattgtct tttttactta tttaaaaatg ttaaaaagaa 67440
ctgagaatta aacatgacct taataactcc aaaagatata ctttatactt tgtgaatgaa 67500
tccgtgtttt aaatagtcca gacagcgctt tacttctttg ttgaatgatt cagttgtttg 67560
acctaatctt ctcactcatt agacattact accacctact ggaagattta gattcttatt 67620
ttgaggatta ttttattttt ttaaaaatat atttttttca tattaaaatg caataaagta 67680
caataaacgt tatttgaaat aaagagatag ttcacacgca caaaattatt ttgtcatcat 67740
atacacactt ttgtgccatt taaatctgta acaatttctt gttattgtgt gttcagtaaa 67800
tgtttacttg attaattctt tttacattta actcaataat gacttgaaat cttctgttag 67860
tggtaatttc tgagtcaatt ttataacatt taacacagaa ttttcccaat agtttgcatt 67920
aattgacttc aggataacta ataatagctt taaagtggcc acctatcttt attttacccc 67980
aggaccataa aaaataaaat tacaatttaa acacacacaa acacacacac acacacacac 68040
acacacacac acacacacac atatatataa agtgtgtgtg tgtgtgtgtg tgtgtgtgtg 68100
tgtgtgtgtg tataaattgt aattttataa ttaatttatt atgtatattt attttattat 68160
atatatatgt gtgtgtgtgt gtgtaaattg taattttata attaatttat tatgtatata 68220
tattttttaa ttatatatat atatatatat atatatatat atatatatat atatatatat 68280
atatatatat atatatatat atatatatat atatatatat atacacacac acacacacac 68340
acacacacac acacannnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 68400
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 68460
atatatatat atatatatat atatatatat atatatatat atatatatat atatatatat 68520
atatatatat atatatatat atatatatat atatatatat atatntatat atatatatat 68580
atatatatat atatatatat atatatatat atatatatat atatatatat atatagtgta 68640
tatatgtata tatatatata tatatatata tatatatata tntatatata tatatatata 68700
tatatatata tatatatata tatatatata tatatatata tatatatata tatatagana 68760
tttttttttt tttttttttt tttttttttt ttcgcttttt ttcgcttttt taagaaggtg 68820
gtttgtcttg gtcgggcatc tcagttgaga cctgtccact atgggtgacc ctaccggtag 68880
ctgtgaagta ccagtggcgt agctctcagc atcactgatg cacacaagcc ctcacagcac 68940

gtcaagctgc aaaccgtgtg agggacccta acctacccta acccaacagc caacactaac 69000
aactctaaca acaaccacta atgtgccaaa aaccactcag aggagaccag caacacgcaa 69060
gaaaccgcct aaacgcatgt caacctacag caaagcatcc aaaagaaccc aagagccgaa 69120
taccaacagc actgacaatc accaaaacac ccagcaatgt gatgaaaagt tgaattgaaa 69180
gtgaaatttg atgtattgta aacttattct ataactattt acaatatata cagtatgtac 69240
atacgtgccc atgatcctcc atctggcact aggatggctc agtgcctggg cttcttcctc 69300
cggggctcct tgggcagcgg ctccttgctg atgtccacca cgatatgagg ggggacactg 69360
gacccggatc tgacctccgt gatgaacaca cgtccatcag gctgctcagt ctctagcagt 69420
gcgggccggt ccagcttctc ccacagccgc tgctttagct ccaatccttg ctgaagagtg 69480
taccggcctg cacgcttctt cacagtggtg tccatcacac tccggtagat ctgctgatat 69540
tcctccacac tccgaccgtg gatggagagg ggctcctcct gtggcctctc acccactggg 69600
gagactgaag ctgcaggacg gagggcagga aggacagccg cgttccgcag gtgtcgctgc 69660
tccacggagg tgtcgcgctc cagcaagaag tccttgaggt gtccaggaag agtcctggtc 69720
cttcttgaac gtcgcagagg catgttgaac aacttcccaa actgatgaaa gtcagccaac 69780
aggagattag ttcagagttt agtagcgcgc tgatcgggat tgataacttt atagacagtt 69840
gtgtagattt gtaaaccaac tcaggttgcc gtggtgacag aatgacgagg aacatttaaa 69900
tttaaacaat aattgatttc agcgttaagt caattagagc agcacgagct gtgtgtttgt 69960
acagtctttg ttattaaata cagaaaatat tttaataatt attaggtata gtaataaagg 70020
gcccatatac ctaaaaaaat aaatattgtc cccgttttac tttaggatag ttttacattg 70080
taaatcgtca tacgtttcca cgtcagaaaa aaacattaat aatgtttaac caataaagta 70140
aaattttagc aatgttacat gacatcttct ttaaccaact aaatgagaca catcagcaca 70200
cattatgctc tcaaggcacg atggtatgaa ggacgaaaac tgcaaaaaca ataaataaat 70260
acgcaaatat ataaacaaat aagtaaataa atccataaat ccctgcataa attaaataat 70320
aaataaataa atatgctaat aaataaataa attgaaaatt ccacaaattc ttgtataaat 70380
aattatttaa ataataaata gtgcgggcag gtaaataatt aaatagccta atttacacga 70440
atgttggggg caataagaaa atgctaaact gatgtttatg tttattcatt tattcacatc 70500
agcatgttga cttgaggaaa agtaaatacg taattatgtt caagaaatga attaacgttt 70560
gaagggggaa ataaactttt acttttcccc ttaacatttg tgtaatttta tatacatttt 70620
atacacattt atttatttgc atatttattt atttattatt ttatttatac agggatttat 70680
gaattattta ctcatttatt tatatatttg cgtatttatt tatttgttgt ttttgcaggt 70740
ttcgtcctcc atacgatggt gagctcattt ttaacaatct ttttaaaatg ttatctaaaa 70800
aatataatgt aaatttcagg atgttttcca gagatttgtg taattttcat ccatatttta 70860
gaaaaaccaa aactgaaaaa cacaaaaacc tgtgcatgta agaatgacaa tttgctttta 70920
gggatacatt tttttctgtg tagctgtcag tgtcgttcag gactatgagg cagtgttgag 70980
agtctttata tctgtgttct aggttgatgt ttgtaagttt tatggaggac agagaagaaa 71040
acctgtacct attcctgaag gcagtgatag tgacagcatt gatcagacaa taaggtaagt 71100
gaggaagaaa ttaatcaggg acagttcact gtaacaggtc gtagtttgtt atggaggtca 71160
ggcaattata ttaatttcct acaccatgtt ttggagatgc gtcttgccat agcagtcaga 71220
ggaggtgcat tctccaggtt actacaagca aatgatttcc tctaagtgtc tctaacacat 71280
tgtccagcaa acaaacgttt actcacttca acaagagatg aactcaccac tgaactgcac 71340
agaaaaagaa atgtggtata gtatccacag ctagtgtttt tcagcctgtg aaagcttgat 71400
gtgactattt ttaattttat aaatcgatgt tgtaatgtaa ttactataca cataagttaa 71460
atagacttaa ccattgtttg aattgtctaa gtgtaaaccg agataaaaga ctgtgtaact 71520
gcaagccccg tcagaatcag taattttaaa gacatggcgg aggaaaatgg aatttaatgc 71580
agcgcttctt gcctggtctg agacccattt caacacttta gaccttagaa attgagattt 71640
acctccagat ccactcttca aaatcagctg tgatgtgacc caaaggggat gttcatataa 71700
ttatttacgt ttttgaggaa ctaatttaat gtataattcc taagaaaaac attgccattc 71760
agttccaaaa cactgcccta aaatagccac agccagtgat gggctgggtt gggtttttgt 71820
taacctgaga atgttctaag ctt 71843

<210> 4
<211> 2261
<212> DNA
<213> Homo sapiens

<220>

```
<221> CDS
<222> (205)...(1965)

<400> 4
ccgcggttcc ggctgctccg gcgaggcgac ccttgggtcg gcgctgcggg cgaggtgggc 60
aggtaggtgg gcggacggcc gcggttctcc ggcaagcgca ggcggcggag tcccccacgg 120
cgcccgaagc gcccccccgca cccccggcct ccagcgttga ggcgggggag tgaggagatg 180
ccgacccaga gggacagcag cacc atg tcc cac acg gtc gca ggc ggc ggc    231
                           Met Ser His Thr Val Ala Gly Gly Gly
                            1               5

agc ggg gac cat tcc cac cag gtc cgg gtg aaa gcc tac tac cgc ggg   279
Ser Gly Asp His Ser His Gln Val Arg Val Lys Ala Tyr Tyr Arg Gly
10                  15                  20                  25

gat atc atg ata aca cat ttt gaa cct tcc atc tcc ttt gag ggc ctt   327
Asp Ile Met Ile Thr His Phe Glu Pro Ser Ile Ser Phe Glu Gly Leu
                30                  35                  40

tgc aat gag gtt cga gac atg tgt tct ttt gac aac gaa cag ctc ttc   375
Cys Asn Glu Val Arg Asp Met Cys Ser Phe Asp Asn Glu Gln Leu Phe
            45                  50                  55

acc atg aaa tgg ata gat gag gaa gga gac ccg tgt aca gta tca tct   423
Thr Met Lys Trp Ile Asp Glu Glu Gly Asp Pro Cys Thr Val Ser Ser
        60                  65                  70

cag ttg gag tta gaa gaa gcc ttt aga ctt tat gag cta aac aag gat   471
Gln Leu Glu Leu Glu Glu Ala Phe Arg Leu Tyr Glu Leu Asn Lys Asp
    75                  80                  85

tct gaa ctc ttg att cat gtg ttc cct tgt gta cca gaa cgt cct ggg   519
Ser Glu Leu Leu Ile His Val Phe Pro Cys Val Pro Glu Arg Pro Gly
90                  95                  100                 105

atg cct tgt cca gga gaa gat aaa tcc atc tac cgt aga ggt gca cgc   567
Met Pro Cys Pro Gly Glu Asp Lys Ser Ile Tyr Arg Arg Gly Ala Arg
                110                 115                 120

cgc tgg aga aag ctt tat tgt gcc aat ggc cac act ttc caa gcc aag   615
Arg Trp Arg Lys Leu Tyr Cys Ala Asn Gly His Thr Phe Gln Ala Lys
            125                 130                 135

cgt ttc aac agg cgt gct cac tgt gcc atc tgc aca gac cga ata tgg   663
Arg Phe Asn Arg Arg Ala His Cys Ala Ile Cys Thr Asp Arg Ile Trp
        140                 145                 150

gga ctt gga cgc caa gga tat aag tgc atc aac tgc aaa ctc ttg gtt   711
Gly Leu Gly Arg Gln Gly Tyr Lys Cys Ile Asn Cys Lys Leu Leu Val
    155                 160                 165

cat aag aag tgc cat aaa ctc gtc aca att gaa tgt ggg cgg cat tct   759
His Lys Lys Cys His Lys Leu Val Thr Ile Glu Cys Gly Arg His Ser
170                 175                 180                 185

ttg cca cag gaa cca gtg atg ccc atg gat cag tca tcc atg cat tct   807
Leu Pro Gln Glu Pro Val Met Pro Met Asp Gln Ser Ser Met His Ser
                190                 195                 200
```

gac cat gca cag aca gta att cca tat aat cct tca agt cat gag agt 855
Asp His Ala Gln Thr Val Ile Pro Tyr Asn Pro Ser Ser His Glu Ser
205 210 215

ttg gat caa gtt ggt gaa gaa aaa gag gca atg aac acc agg gaa agt 903
Leu Asp Gln Val Gly Glu Glu Lys Glu Ala Met Asn Thr Arg Glu Ser
220 225 230

ggc aaa gct tca tcc agt cta ggt ctt cag gat ttt gat ttg ctc cgg 951
Gly Lys Ala Ser Ser Ser Leu Gly Leu Gln Asp Phe Asp Leu Leu Arg
235 240 245

gta ata gga aga gga agt tat gcc aaa gta ctg ttg gtt cga tta aaa 999
Val Ile Gly Arg Gly Ser Tyr Ala Lys Val Leu Leu Val Arg Leu Lys
250 255 260 265

aaa aca gat cgt att tat gca atg aaa gtt gtg aaa aaa gag ctt gtt 1047
Lys Thr Asp Arg Ile Tyr Ala Met Lys Val Val Lys Lys Glu Leu Val
270 275 280

aat gat gat gag gat att gat tgg gta cag aca gag aag cat gtg ttt 1095
Asn Asp Asp Glu Asp Ile Asp Trp Val Gln Thr Glu Lys His Val Phe
285 290 295

gag cag gca tcc aat cat cct ttc ctt gtt ggg ctg cat tct tgc ttt 1143
Glu Gln Ala Ser Asn His Pro Phe Leu Val Gly Leu His Ser Cys Phe
300 305 310

cag aca gaa agc aga ttg ttc ttt gtt ata gag tat gta aat gga gga 1191
Gln Thr Glu Ser Arg Leu Phe Phe Val Ile Glu Tyr Val Asn Gly Gly
315 320 325

gac cta atg ttt cat atg cag cga caa aga aaa ctt cct gaa gaa cat 1239
Asp Leu Met Phe His Met Gln Arg Gln Arg Lys Leu Pro Glu Glu His
330 335 340 345

gcc aga ttt tac tct gca gaa atc agt cta gca tta aat tat ctt cat 1287
Ala Arg Phe Tyr Ser Ala Glu Ile Ser Leu Ala Leu Asn Tyr Leu His
350 355 360

gag cga ggg ata att tat aga gat ttg aaa ctg gac aat gta tta ctg 1335
Glu Arg Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp Asn Val Leu Leu
365 370 375

gac tct gaa ggc cac att aaa ctc act gac tac ggc atg tgt aag gaa 1383
Asp Ser Glu Gly His Ile Lys Leu Thr Asp Tyr Gly Met Cys Lys Glu
380 385 390

gga tta cgg cca gga gat aca acc agc act ttc tgt ggt act cct aat 1431
Gly Leu Arg Pro Gly Asp Thr Thr Ser Thr Phe Cys Gly Thr Pro Asn
395 400 405

tac att gct cct gaa att tta aga gga gaa gat tat ggt ttc agt gtt 1479
Tyr Ile Ala Pro Glu Ile Leu Arg Gly Glu Asp Tyr Gly Phe Ser Val
410 415 420 425

gac tgg tgg gct ctt gga gtg ctc atg ttt gag atg atg gca gga agg 1527

Asp Trp Trp Ala Leu Gly Val Leu Met Phe Glu Met Met Ala Gly Arg
430 435 440

tct cca ttt gat att gtt ggg agc tcc gat aac cct gac cag aac aca 1575
Ser Pro Phe Asp Ile Val Gly Ser Ser Asp Asn Pro Asp Gln Asn Thr
445 450 455

gag gat tat ctc ttc caa gtt att ttg gaa aaa caa att cgc ata cca 1623
Glu Asp Tyr Leu Phe Gln Val Ile Leu Glu Lys Gln Ile Arg Ile Pro
460 465 470

cgt tct ctg tct gta aaa gct gca agt gtt ctg aag agt ttt ctt aat 1671
Arg Ser Leu Ser Val Lys Ala Ala Ser Val Leu Lys Ser Phe Leu Asn
475 480 485

aag gac cct aag gaa cga ttg ggt tgt cat cct caa aca gga ttt gct 1719
Lys Asp Pro Lys Glu Arg Leu Gly Cys His Pro Gln Thr Gly Phe Ala
490 495 500 505

gat att cag gga cac ccg ttc ttc cga aat gtt gat tgg gat atg atg 1767
Asp Ile Gln Gly His Pro Phe Phe Arg Asn Val Asp Trp Asp Met Met
510 515 520

gag caa aaa cag gtg gta cct ccc ttt aaa cca aat att tct ggg gaa 1815
Glu Gln Lys Gln Val Val Pro Pro Phe Lys Pro Asn Ile Ser Gly Glu
525 530 535

ttt ggt ttg gac aac ttt gat tct cag ttt act aat gaa cct gtc cag 1863
Phe Gly Leu Asp Asn Phe Asp Ser Gln Phe Thr Asn Glu Pro Val Gln
540 545 550

ctc act cca gat gac gat gac att gtg agg aag att gat cag tct gaa 1911
Leu Thr Pro Asp Asp Asp Asp Ile Val Arg Lys Ile Asp Gln Ser Glu
555 560 565

ttt gaa ggt ttt gag tat atc aat cct ctt ttg atg tct gca gaa gaa 1959
Phe Glu Gly Phe Glu Tyr Ile Asn Pro Leu Leu Met Ser Ala Glu Glu
570 575 580 585

tgt gtc tgatcctcat ttttcaacca tgtattctac tcatgttgcc atttaatgca 2015
Cys Val

tggataaact tgctgcaagc ctggatacaa ttaaccattt tatatttgcc acctacaaaa 2075
aaacacccaa tatcttctct tgtagactat atgaatcaat tattacatct gttttactat 2135
gaaaaaaaaa ttaatactac tagcttccag acaatcatgt caaaatttag ttgaactggt 2195
ttttcagttt ttaaaaggcc tacagatgag taatgaagtt accttttttg tttaaaaaaa 2255
aaaaag 2261

<210> 5
<211> 587
<212> PRT
<213> Homo sapiens

<400> 5
Met Ser His Thr Val Ala Gly Gly Gly Ser Gly Asp His Ser His Gln
1 5 10 15
Val Arg Val Lys Ala Tyr Tyr Arg Gly Asp Ile Met Ile Thr His Phe

20 25 30
Glu Pro Ser Ile Ser Phe Glu Gly Leu Cys Asn Glu Val Arg Asp Met
35 40 45
Cys Ser Phe Asp Asn Glu Gln Leu Phe Thr Met Lys Trp Ile Asp Glu
50 55 60
Glu Gly Asp Pro Cys Thr Val Ser Ser Gln Leu Glu Leu Glu Glu Ala
65 70 75 80
Phe Arg Leu Tyr Glu Leu Asn Lys Asp Ser Glu Leu Leu Ile His Val
85 90 95
Phe Pro Cys Val Pro Glu Arg Pro Gly Met Pro Cys Pro Gly Glu Asp
100 105 110
Lys Ser Ile Tyr Arg Arg Gly Ala Arg Arg Trp Arg Lys Leu Tyr Cys
115 120 125
Ala Asn Gly His Thr Phe Gln Ala Lys Arg Phe Asn Arg Arg Ala His
130 135 140
Cys Ala Ile Cys Thr Asp Arg Ile Trp Gly Leu Gly Arg Gln Gly Tyr
145 150 155 160
Lys Cys Ile Asn Cys Lys Leu Leu Val His Lys Lys Cys His Lys Leu
165 170 175
Val Thr Ile Glu Cys Gly Arg His Ser Leu Pro Gln Glu Pro Val Met
180 185 190
Pro Met Asp Gln Ser Ser Met His Ser Asp His Ala Gln Thr Val Ile
195 200 205
Pro Tyr Asn Pro Ser Ser His Glu Ser Leu Asp Gln Val Gly Glu Glu
210 215 220
Lys Glu Ala Met Asn Thr Arg Glu Ser Gly Lys Ala Ser Ser Ser Leu
225 230 235 240
Gly Leu Gln Asp Phe Asp Leu Leu Arg Val Ile Gly Arg Gly Ser Tyr
245 250 255
Ala Lys Val Leu Leu Val Arg Leu Lys Lys Thr Asp Arg Ile Tyr Ala
260 265 270
Met Lys Val Val Lys Lys Glu Leu Val Asn Asp Asp Glu Asp Ile Asp
275 280 285
Trp Val Gln Thr Glu Lys His Val Phe Glu Gln Ala Ser Asn His Pro
290 295 300
Phe Leu Val Gly Leu His Ser Cys Phe Gln Thr Glu Ser Arg Leu Phe
305 310 315 320
Phe Val Ile Glu Tyr Val Asn Gly Gly Asp Leu Met Phe His Met Gln
325 330 335
Arg Gln Arg Lys Leu Pro Glu Glu His Ala Arg Phe Tyr Ser Ala Glu
340 345 350
Ile Ser Leu Ala Leu Asn Tyr Leu His Glu Arg Gly Ile Ile Tyr Arg
355 360 365
Asp Leu Lys Leu Asp Asn Val Leu Leu Asp Ser Glu Gly His Ile Lys
370 375 380
Leu Thr Asp Tyr Gly Met Cys Lys Glu Gly Leu Arg Pro Gly Asp Thr
385 390 395 400
Thr Ser Thr Phe Cys Gly Thr Pro Asn Tyr Ile Ala Pro Glu Ile Leu
405 410 415
Arg Gly Glu Asp Tyr Gly Phe Ser Val Asp Trp Trp Ala Leu Gly Val
420 425 430
Leu Met Phe Glu Met Met Ala Gly Arg Ser Pro Phe Asp Ile Val Gly
435 440 445
Ser Ser Asp Asn Pro Asp Gln Asn Thr Glu Asp Tyr Leu Phe Gln Val
450 455 460
Ile Leu Glu Lys Gln Ile Arg Ile Pro Arg Ser Leu Ser Val Lys Ala
465 470 475 480
Ala Ser Val Leu Lys Ser Phe Leu Asn Lys Asp Pro Lys Glu Arg Leu

```
                    485                                     490                                     495
Gly Cys His Pro Gln Thr Gly Phe Ala Asp Ile Gln Gly His Pro Phe
                500                                     505                                     510
Phe Arg Asn Val Asp Trp Asp Met Met Glu Gln Lys Gln Val Val Pro
            515                                     520                                     525
Pro Phe Lys Pro Asn Ile Ser Gly Glu Phe Gly Leu Asp Asn Phe Asp
    530                                     535                                     540
Ser Gln Phe Thr Asn Glu Pro Val Gln Leu Thr Pro Asp Asp Asp Asp
545                                     550                                     555                                     560
Ile Val Arg Lys Ile Asp Gln Ser Glu Phe Glu Gly Phe Glu Tyr Ile
                    565                                     570                                     575
Asn Pro Leu Leu Met Ser Ala Glu Glu Cys Val
                580                                     585
```

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 March 2003 (20.03.2003)

PCT

(10) International Publication Number
WO 2003/023048 A3

(51) International Patent Classification[7]: C07K 16/00, 17/00, A01K 67/033, A61K 38/00, 31/70, C07H 21/04, A61K 49/00

(21) International Application Number: PCT/US2002/028410

(22) International Filing Date: 6 September 2002 (06.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/317,653 6 September 2001 (06.09.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/317,653 (CIP)
Filed on 6 September 2001 (06.09.2001)

(71) Applicant (*for all designated States except US*): **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **PETERSON, Randall** [US/US]; 42 Perkins Street, Stoneham, MA 02180 (US). **FISHMAN, Mark, C.** [US/US]; 43 Kenwood Avenue, Newton Center, MA 02459 (US).

(74) Agent: **MICHAUD, Susan, M.**; Clark & Elbing LLP, 101 Federal Street, Boston, MA 02110 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

(88) Date of publication of the international search report: 15 January 2004

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 2003/023048 A3

**(54) Title:** METHODS FOR DIAGNOSING AND TREATING DISEASES AND CONDITIONS ASSOCIATED WITH PROTEIN KINASE Cλ

**(57) Abstract:** The invention provides methods of diagnosing diseases and conditions associated with PKCλ, methods for identifying compounds that can be used to treat or to prevent such diseases and conditions, and methods of using these compounds to treat or to prevent such diseases and conditions. Also provided in the invention are animal model systems that can be used in screening methods.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/28410

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC(7) : C07K 16/00; C07K 17/00; A01K 67/033; A61K 38/00; A61K 31/70; C07H 21/04 ; A61K 49/00
US CL : 530/387.1; 530/350; 800/13; 514/2; 514/44; 536/23.1;424/9.2
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/387.1; 530/350; 800/13; 514/2; 514/44; 536/23.1;424/9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 2003/0017969 A1 (TENNENBAUM et al.) 23 January 2003 (23.01.2003), page 2, para. 0013-0015; page 3, para. 001431; page 17, para. 0171. | 12,15,16 |
| X,P | HORNE-BADOVINAC, S. et al. Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. Current Biology, October 2001, Vol. 11, pages 1492-1501, see "Gnetic mapping and positional cloning and reference to Genbank Accession #AF390109. | 21-25 |
| X<br>---<br>Y | AKIMOTO et al. A new member oif the third class in the protein kinase C family, PKC-lambda, expressed dominantly in an undifferentiated mouse ebryonal carcinoma call line and also in many tissues and cells. Jour. Biol. Chem., 29 April 1994, Vol. 17, pages 12677-12681, see column 2, lines 8-10 and Figure 1B. | 32<br>----------<br>27,28, 33 |
| Y | WANG et al. Expression of a dominant negative type II transforming growth factor beta (TGF-beta) receptor in the epidermis of transgenic mice blocks TGF-beta-mediated growth inhibition. PNAS, March 1997, Vol. 94, pages 2386-2391. | 29 |
| Y | BANDYOPADHAYA et al. Effects of adenoviral gene trasnfer of wild-type, constitutively active, and kinase-defective protein kinase C-lambda on insulin-stimulated glucose transport in L6 myotubes. Endocrinology, 2000, Vol. 141, No. 11, pages 4120-4127. | 29 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| | |
|---|---|
| Date of the actual completion of the international search<br>12 February 2003 (12.02.2003) | Date of mailing of the international search report<br>31 OCT 2003 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703)305-3230 | Authorized officer<br>Valarie Bertoglio<br>Felicia D. Roberts for<br>Telephone No. 703-308-1234 |